# The Physiology and Pathology of Hemostasis

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*With 18 Illustrations*

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*To my wife Margaret*
*and daughter Edith*

# PREFACE

TREMENDOUS strides have been made in the past decade in the study of the coagulation of the blood and in the broad subject of hemostasis. Clinicians and physiologists who have only a casual acquaintance with this field are becoming bewildered at the radical changes that are occurring in the basic concepts as well as by the new terms that are being continuously introduced. The writer has had the temerity to assemble the facts and findings garnered from clinical and from purely scientific researches in hemostasis, and to construct out of them a concept that is more in accord with the known physiological principles than are the views that have dominated thinking since 1731 when Petit introduced the basic idea that bleeding is controlled by a fibrin clot which functions as a mechanical plug or seal, or as a recent writer has expressed it, "the gel that keeps a cut person from bleeding to death."

The new hypothesis to explain hemostasis which is epitomized in figure 2 is not presented with any sense of finality, but is offered as a working blueprint, which will require changes and modifications, as new knowledge becomes available. Even in its present form, it serves in unifying the concept of hemostasis, in outlining a logical classification of the hemorrhagic diseases and in placing therapy on a rational basis. A chapter is devoted to each of these objectives. Since the new hypothesis applies also to venous thrombosis, it was felt desirable to include a chapter on this subject.

Most of the important advances in hemostasis have resulted from laboratory studies. Quantitative procedures for estimating the various agents participating in the clotting mechanism are indispensable in the study and management of the hemorrhagic diseases. In the second half of the monograph, therefore, the laboratory aspects of the subject are presented. The tests and procedures currently employed in the writer's laboratory are described in detail and are critically discussed.

The author is sensible of the assistance he has obtained from his associates. During the past five years a number of young physicians, Doctors Mario Stefanini, Rene Honorato, Jacob Shanberge, Jean Favre-Gilly, and George Collentine have worked with the author, and have made valuable contributions, many of which are presented in this monograph Miss Clara Hussey and Mr. Ernest Epstein have rendered valuable service, especially in testing and modifying methods Mr. William Stapp, a hemophiliac who is now a Senior medical student, has in the five years spent with the writer supplied valuable information on hemophilia. The illustrations were made by Mr Leo Massopust. To him and to others of the faculty of Marquette University School of Medicine, the author expresses his gratitude for their cooperation. The clinical opportunities which the writer is enjoying through his association with Milwaukee Children's Hospital are exceedingly helpful for him to maintain a balanced point of view For the past four years the studies on hemophilia have been aided by grants from the United States Public Health Service, which are gratefully acknowledged

The author is appreciative of many courtesies of the publisher, Lea & Febiger A number of the charts and tables used in this monograph appeared previously in The American Journal of the Medical Sciences

Ever mindful of Osler's well-known dictum "To study the phenomenon of disease without books is to sail an unchartered sea, while to study books without patients is not to go to sea at all," the author hopes that this little volume will be of help to those who venture on the troubled waters of the hemorrhagic diseases

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MILWAUKEE, WISCONSIN

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## Chapter 1

## THE HISTORICAL DEVELOPMENT OF THE CONCEPTS OF HEMOSTASIS

It is inborn in man to accept the bounties of nature and the contributions of his fellow men as a matter of course. To this the surgeon is no exception. Equipped with the tampon, the ligature and the hemostat, the surgeon so successfully achieves hemostasis, that it rarely occurs to him that even such a simple tool as the ligature did not always exist He probably does not know that it was perhaps first used by an unknown physician in the Alexandrian era, that it was forgotten for nearly a thousand years and then rediscovered by Ambroise Paré

It is easy to overlook the fact that with every incision, innumerable capillaries, venules and arterioles are severed, because stanching occurs so automatically It is not surprising, therefore, that the subject of hemostasis attracts relatively little attention, and that most modern standard textbooks of surgery barely mention this important physiological mechanism Nevertheless, a function that is so perfected that it can cope both with the onslaught of man and the unfavorable environment resulting from disease, makes it all the more worthy of intensive study

### VASCULAR CONTRACTION

No thorough understanding of hemostasis can be attained without a broad knowledge of its historical development Until Harvey's monumental discovery of the circulation of the blood, all explanations of the control of hemorrhage were purely speculative Petit[74] (1731) must be credited for the first scientific approach to the physiology of hemostasis He concluded that hemorrhage is stopped by the formation of a coagulum of blood He recognized that part of the clot was in the vessel and to this he gave the descriptive name bouchon (cork), and part was outside which he designated as couvercle (cover) He

observed the adherence of clots to the internal coat of the vessel. Five years later (1736) Morand[65] offered a second type of explanation. He postulated that an artery could undergo a longitudinal contraction resulting in corrugation or plaiting whereby the lumen was diminished. While Morand's hypothesis was anatomically faulty since the muscle fibers of arteries are circular and not longitudinal, he did introduce the important concept of contraction as a factor in hemostasis With the annunciation of these two explanations for the control of hemorrhage, a controversy was initiated which remains unsettled even today. A number of the leading surgeons in England including Sharp,[90] Gooch,[27] Kirkland[47] and White[109] accepted the contraction theory in preference to Petit's hypothesis It is interesting to recall that John Hunter[39] was particularly interested in traumatic arterial spasm and stated that an injured vessel has a natural disposition to contract. It remained for Jones[43] (1805) to present a concept of hemostasis which combined the divergent views, and disclosed a remarkable clarity of view He stated "We can no longer consider the suppression of hemorrhage as a simple or mechanical effect, but as a process performed by the concurrent and successive operations of many causes these may be simply stated to consist in the retraction and contraction of the artery, the formation of a coagulum at its mouth, the inflammation and consolidation of its extremity by an effusion of coagulating lymph within its canal, between its tunic and in the cellular substance surrounding it "

In the nineteenth century especially during the latter half, the developments in the field of coagulation of the blood dominated and the contraction theory was either ignored or forgotten The marked shift to Petit's original concept of hemostasis is illustrated by the fact that such eminent surgeons as Joseph Lister[56] not only upheld the coagulation theory but contributed actively to its advancement

In 1923 Magnus[60] again brought the contraction theory to the foreground He showed that normal arteries on injury can retract so intensely that the lumen is obliterated and the flow of blood is completely stopped He concluded that "the process of hemostasis must be considered more than heretofore as a property of the vessel itself and that the coagulation of blood is not the only, perhaps not even the essential factor in bringing about stanching" (Author's translation) Interestingly enough Morawitz,[68] who in 1904 formulated the classical theory of coagulation, markedly broadened his concept of hemostasis twenty years later as evidenced by two of his statements

"Blood coagulation is not the only factor which controls hemorrhage, yes, perhaps not even the most important"

"Thus, we see that for the control of bleeding one must regard blood

and the vessel as interacting in an inseparable linked unity, and that it is just as erroneous to attribute all anomalies (of hemostasis) to the blood as to consider only the vessel " (Author's translation )

Similar views were expressed by Schulz,[89] Stich[97] and others. Kuttner and Baruch[49] on the basis of the study of an extensive series of battlefield injuries emphasized segmental spasm of injured arteries, which they found was sometimes so intense that the severed free end of the vessel became pulseless Stegemann,[92] although recognizing the importance of both contraction and coagulation, introduced and emphasized an additional factor in hemostasis, namely a shunting of the blood flow away from the site of injury. Tannenberg and Hermann[99] as well as others attacked this hypothesis The clearest and no doubt final evaluation of shunting is given by Apitz[3] who stated: "The deflection of blood from an injured vessel is always the result; never the cause of stanching " (Author's translation )

Tannenberg and Hermann were perhaps the first to emphasize clearly that purely mechanical factors may participate in hemostasis According to Tannenberg and his students there is in addition to the physiological contraction, a retraction of the cut artery due to tension in and about the vessel and also the pressure exerted by the hematoma formed at the site of injury Oddly enough this phase of hemostasis has received little attention Recently, however, Tocantins[100] has critically and illuminatingly discussed these mechanical extravascular factors from the point of view of clinical application

Even more important than the pressure or force that closes the vessel, is the consequence when intimal endothelial cells are pressed together They develop a stickiness which results in a gluing action [illegible] ves- [illegible] the [illegible] "The strong contraction presses the intimal surfaces together for a considerable time and brings about a closure by adhesion " (Author's translation ) Herzog[32] concluded on the basis of studies on the capillaries of the frog's tongue that bleeding of capillaries is controlled in cold blooded animals primarily by adhesion Roskam[84] postulated that this increased stickiness of irritated or injured endothelial cells is a type of opsonization brought about by the precipitation of an adhesive protein Morawitz[68] also recognized the importance of this capillary adhesiveness in hemostasis and Apitz[3] recently again discussed this mechanism He believed that it could be better explained on the basis of simple mechanical pressure, than by the more elaborate hypothesis of Roskam Very recently Chen and Tsai[15] concluded that the capillary bleeding is controlled by adhesion of its

walls as a consequence of endothelial injury. Chen and Tsai were apparently not aware of the earlier work, for they state. "As the phenomenon of capillary adhesion after mechanical injury is so constant and persistent, it is difficult to explain why it has escaped the attention of previous workers" In a subsequent paper, they cite the observations of other investigators on capillary adhesions and add their own important finding that the adhesive force of the capillary of the toad may withstand a pressure of 200 mm. of mercury. The contraction of capillaries as a means of controlling hemorrhage remains difficult to evaluate. Chen and Tsai point out the complexity of the problem since capillary contractility varies according to species and situation Macfarlane,[57] however, centers his concept of hemostasis on the vascular response to injury.

## THE COAGULATION OF THE BLOOD—HISTORICAL HIGHLIGHTS

Experimental work on coagulation of the blood began three centuries ago and among the investigators are included the names of many illustrious men in medicine and in science. The development in this field may be divided into three periods (1) the pre-classical theory era, (2) the classical theory era, and (3) the prothrombin era

**The Pre-Classical Theory Era (1666-1904).**—The observation of Malpighi[61] in 1666 that strands of fibers remained after a clot of blood was washed can be regarded as the first attempt to gain an insight into the mysterious power of the blood to clot A century later, Hewson[33] developed the first methods for keeping blood fluid outside of the body He thereby laid the foundation for nearly all further study of coagulation. As a result of his investigation, it was established that the coagulation factors resided in the non-cellular moiety of the blood Buchanan[12] in 1835 offered an explanation of the coagulation reaction which was based on laboratory studies It anticipated some of the cardinal features of the classical theory Buchanan compared the clotting of blood with the curdling of milk by rennin In both, a ferment reacted with a soluble protein to produce a coagulum Although Buchanan's work was apparently well-known in England, as evidenced by the fact that Lister[56] used his data as a basis for his own studies in 1863, it failed, nevertheless, to stimulate others and thus studies on coagulation declined in England, and a period of great activity began on the Continent Hammarsten[29] (1877) contributed valuable information on fibrinogen, and was the first to state that only thrombin was necessary to coagulate this protein, while Schmidt[87] after many years of study formulated a logical scheme to

explain the process of coagulation. He postulated that thrombin was formed from a precursor (named prothrombin by Pekelharing) which required for its activation a zymoplastic agent. In 1890 Arthus and Pagès[5] showed by irrefutable evidence that calcium was essential for coagulation. It now remained for Morawitz[64] and Fuld and Spiro[35] in 1904 to assemble the data and condense it to the two well-known equations

Prothrombin + $Ca^{++}$ + Thrombokinase = Thrombin
Fibrinogen + Thrombin = Fibrin

With the formulation of this hypothesis now commonly called the classical theory, a new era had its beginning

In the period that ended in 1904, only a few clinical advances occurred. Most notable were the recognition of hemorrhagic purpura by Werlhof[106] in 1735 and hemophilia by Otto[73] in 1803. Oddly enough it took nearly a century before the prolonged coagulation in the disease was recognized, but this is not surprising for it was not until in 1878 that Vierordt[103] devised the first method for determining coagulation time. It has already been pointed out that Petit considered the blood coagulum as the primary means whereby the body effected hemostasis, and this view, although it met early opposition, steadily gained, and continues to dominate even today

**The Classical Theory Era (1904-1934).**—The simple concept expressed by the two equations of Morawitz paradoxically did not lead to agreement and unity among investigators, but rather to a multiplicity of theories Only a few can be mentioned. Nolf[71] postulated

glass, coagulating lipoids, and chloroform were held to aid the reaction and, therefore, called thromboplastic substance Thrombin was considered not as the clotting agent, but as unsaturated fibrin produced by

from platelets and body tissues

He postulated that serozyme was present in blood as a precursor, proserozyme In the presence of calcium and on contact with a rough surface it became activated and then reacted with cytozyme (thromboplastin) to form thrombin These theories hampered rather than advanced the study of coagulation This does not gainsay that these

concepts, especially that of Bordet, contained ideas which modern *research is substantiating at least in part, but when they were enunciated* they confused rather than clarified the issues. It was the classical theory which remained the most reliable guide and led to the noteworthy achievements that began in 1934.

A number of important clinical advances were made in this period. The introduction of sodium citrate as an anticoagulant independently by Hustin,[40] Agate[2] and Lewisohn[54] in 1915, when judged by the number of lives saved by blood and plasma transfusions, can be ranked among the important medical contributions of all times. The bleeding time test introduced by Duke[19] in 1912 supplied the clinical laboratory with a new tool for a field of study that had a paucity of laboratory aids The discovery of Kaznelson[45] in 1916 that splenectomy abolished the bleeding diathesis in essential thrombocytopenic purpura, was the first instance in which a hemorrhagic disease was *cured by a specific means The discovery of heparin by McLean[63] in* 1916 and its subsequent study by Howell[34, 35] gave to medicine a new tool and a valuable therapeutic agent

*These practical contributions greatly outranked the theoretical* advances made in hemostasis As already stated the period between 1904 and 1934 was one of confusion which led to much desultory research The clinician and surgeon had little in common with the physiologist The endless conflicts of theories made the physician ignore or actually shy from opinions of the laboratory workers This period was a lull between the intense activity that produced the classical theory and the new era that began in 1934

**The Prothrombin Era.** – *In 1934 began, as Quick has called it,* a renaissance in the study of the coagulation of the blood In seeking the basic cause for these significant and pyramiding advances, one is inclined a priori to attribute them to the discovery of vitamin K While this agent did spectacularly eradicate two mysterious diseases, cholemic bleeding and the hemorrhagic disease of the newborn, it has in the decade and a half since its isolation contributed relatively little towards solving the problem of coagulation Its own action in the sy*nthesis of prothrombin remains unanswered*

Of far greater theoretical importance in advancing the study of coagulation was the development of the simple procedure by Quick[78, 80] and the two-stage method of Warner, Brinkhouse and Smith[101] for estimating prothrombin activity in blood Much of the confusion rampant in the previous era *can be traced to lack of the quantitative* approach The physiologist of necessity had to attack most of his early problems qualitatively This unfortunately often established permanent habits. This is perhaps why biologists often until recently

specified drops for measuring their reagents. The pipette apparently had not become an everyday tool in physiological laboratories

How the lack of a readily available procedure for determining prothrombin hampered progress can easily be demonstrated. In 1912 Whipple[107] reported the absence of prothrombin in a case of melena neonatorum and the following year found that this clotting factor was diminished in a case of jaundice [108] These isolated observations were soon forgotten because only a skilled and able investigator could demonstrate the defect. Likewise Roderick[81] in 1931 showed by a qualitative method that prothrombin was diminished in spoiled sweet clover disease of cattle, but his findings apparently did not assist him or others who sought to isolate the toxic principle Only after the one-stage method was developed, and employed as a means of assaying the extracts of spoiled sweet clover hay was it possible as Link[45] expressed it, "to lay the hemorrhagic agent on the table in pure crystalline form."

By means of the one-stage method Quick, Stanley-Brown and Bancroft[6,78] observed that prothrombin was frequently diminished in jaundiced patients Later, when Quick[79] developed vitamin K deficiency in chicks by Almquist's procedure and found that the prothrombin was diminished and that bleeding resulted when it reached a critical level, he was able to postulate that the bleeding in obstructive jaundice was due to a vitamin K deficiency resulting from faulty intestinal absorption The correctness of his suggestion that vitamin K and bile salts be given therapeutically to counteract the hypoprothrombinemia was promptly verified clinically by Warner and his associates[105] and by Butt and his co-workers [11]

Without discussing the relative merits and defects of the one-stage and two-stage methods, it is easy to understand why the one became a routine clinical test both in America and abroad, while the other was used little except in pure research The one-stage procedure was simple, and the clinician found it reliable both diagnostically and for the control of Dicumarol therapy Oddly enough, the apparent deflections of the method which have been the targets for criticism, have turned out to be due to complexities of prothrombin and actually have led to the discovery of new coagulation factors

*In defense of the two-stage method it should be said that it yielded* in the hands of its originators valuable results The fall in prothrombin after biliary fistula[92] and after experimental liver injury,[91] and the low prothrombin in a case of hemorrhagic disease of the newborn[10] were first demonstrated by this method Had it not been for the rapid headway made by the one-stage test, it would undoubtedly have

occupied a more important position and would have found more extensive employment in the clinical laboratories

Both methods were based on the classical theory. The action of vitamin K, likewise, could be most satisfactorily explained by this theory As a consequence the theory reached its zenith about 1940 and appeared to have secured a permanent and leading position But the discovery in 1943 of a new factor[77] essential for prothrombin activity marked the beginning of its dissolution It had served its purpose well, it made possible a rational classification of the hypoprothrombinemic diseases, it aided in placing therapy on a scientific basis and it made prognosis by means of laboratory tests more reliable

The two outstanding clinical contributions in this era were the introduction of vitamin K and Dicumarol into therapy. The latter resulted from the outstanding researches of Link and his students on spoiled sweet clover hay The elucidation of the structure of vitamin $K_1$ and $K_2$ by Doisy, Almquist, Fieser, Karrer and their associates and the synthesis of the natural vitamin $K_1$ were brilliant chemical achievements, but had no great clinical influence. Of far greater practical value was the work of the Connaught Laboratories at the University of Toronto and the contributions of the Caroline Institute of Stockholm, Sweden, on the purification of heparin, which resulted in a product that could be given by injection with safety.

## THE PLATELETS

It is likely that no other body constituent has received such indifferent or hostile treatment from investigators as the platelet Although Donne[19] in 1842 recognized that there was in addition to red and white cells a third type of cell which he called "les globulins," and which were without question the platelets, their very existence was still questioned during the early part of the present century Starling's "Principles of Human Physiology" in the earlier editions contained a statement that they may be artefacts The greatest difficulty was the establishment of the platelet as a distinct cell independent of the other types of formed elements occurring in the blood A number of the early workers, particularly Zimmermann[113] (1846), Schultze[83] (1865) and especially Hayem[31] considered them either derived from other blood cells or precursors of them Hayem named them hematoblasts *because he believed they were precursors of the* red cell

In 1882 Bizzozero[7] in a brilliant paper based on a study of platelets in the circulating blood not only showed that they were a distinct

type of cell, but that they possessed a peculiar behavior of agglutinating and adhering to foreign or rough surfaces Bizzozero's work was immediately attacked, especially by Schmidt's students, Hegl, Rauschenbach and Feiertag who maintained that the platelets were disintegration products of other blood cells and they opposed the idea that they played a rôle in coagulation as Bizzozero had postulated. Löwit's (1885) views were even more hostile. He postulated that platelets were not even disintegrated fragments of other cells, but precipitated globulin Two years later Wooldridge espoused a similar concept. *The underestimation of the importance of the platelet in* hemostasis by many British investigators, even up to the present time, can probably be traced to the influence of Wooldridge. The account of the clash between Bizzozero and his contemporaries is presented in detail by Burker [15]

The studies of Schimmelbusch,[86] Burker[15] and others who, like Bizzozero, observed the platelets directly in the blood stream, effecttively established these cells as definite entities Further support came from Mosen's[70] demonstration that platelets could be isolated from oxalated plasmas as discrete bodies The most decisive contribution, however, was that of Wright,[110] who convincingly showed that platelets were formed in the bone marrow from the fragmentation of the cytoplasm of the megakaryocytes The events leading to this significant discovery are critically presented by Tocantins [101]

Since it required over half a century to establish that the platelet was an independent type of cell, it is not surprising that the problem of determining its function presented even more formidable difficulties It was gradually recognized that it participates in three hemostatic mechanisms thrombus formation, coagulation and vasoconstriction

**Thrombus Formation.**—Zimmermann[111] as early as 1846 noted the tendency of the new type of cell which he had discovered (Elementarblaschen) to clump The same observation was recorded by Schultze[85] in 1865 Wharton Jones[44] studying the circulating blood of frogs under the microscope had also described the aggregation of colorless corpuscles in little heaps and noted that this mass of cells together with coagulated fibrin sometimes blocked the blood flow in an artery Mantegazza[62] four years later paved the way for a rational explanation for the formation of white thrombi He found that a massing of the little white blood cells occurred about a thread introduced into the blood cell and that a similar piling up of these cells took place at the site of an injured vessel wall This agglutination preceded the appearance of fibrin Zahn[112] independently made similar observations and reached somewhat similar conclusions. He

found that when he cut a vessel, bleeding was stopped not by coagulation but by a white thrombus

The outstanding work of Hayem and Bizzozero left no doubt about the nature of the white thrombus and its function as a hemostatic plug Hayem designated it as "clou hemostatique" while the Germans coined a similar term, "Blutstillenden Keil"

Schimmelbusch[88] contributed two important facts concerning the formation of white thrombi, namely, the stickiness of platelets, and the importance of slowing of the circulation Platelets because of their lightness travel in the center of the blood stream and they only come to the periphery when the circulation becomes sluggish. Schimmelbusch was, therefore, the first to emphasize stasis as a factor in thrombosis

Without attempting to present subsequent researches directed towards this phase of hemostasis, it can be stated that the idea of a platelet plug as a means of controlling bleeding in small vessels has been generally accepted. Macfarlane[57,58] is one of the few modern students of hemorrhagic diseases who holds that the platelets play a subordinate position in hemostasis and that the control of hemorrhage from small vessels is brought about primarily by the contraction of the vessel

**Platelets' Rôle in Coagulation.** —Perhaps no phase of the coagulation problem has remained as unsettled as the rôle of the platelets in coagulation Hayem[31] in 1878 clearly postulated that the platelets were essential to coagulation when he stated "We do not believe that this fibrin can appear unless the hematoblasts (platelets) in undergoing changes give up to the plasma a substance which the latter lacks and without which it cannot coagulate" (Author's translation) Bizzozero[7] reached a similar conclusion "They (the platelets) yield, as a result of the change which they undergo, a substance which acts on the coagulation factor in such a way that it brings about the formation of fibrin" (Author's translation) These views met a hostile reception by Schmidt and his students, and by Lowit, Wooldridge and Schimmelbusch But Kemp[48] at Johns Hopkins in a paper published in 1884, which unfortunately appears to have been entirely forgotten, came to the same conclusions as Hayem and Bizzozero He wrote "The breakdown of the plaques is intimately connected, in its time relation at least, with the clotting of the blood The connection between the break down of the plaques and the coagulation of the blood is not histological but chemical, *i e*, the plaques appear to give up a soluble substance which is active in coagulation The active agent in question is most probably fibrin ferment"

In 1904, Bürker in a masterful study convincingly defended the rôle of platelets in coagulation and formulated an observation which time has shown to have the authenticity of a law: "One observes that every time coagulation is prevented, the disintegration of platelets is prevented; when coagulation is delayed, disintegration is delayed. And when coagulation is not influenced, disintegration is not influenced" (Author's translation)

The findings of Delezenne[18] that the removal of thrombocytes from goose blood by centrifugation, made it incoagulable, as well as the observation of Tait and Green[93] that filtering out spindle cells from frog blood by means of animal charcoal had a similar effect, strengthened the hypothesis that coagulation required a cellular factor The relationship of thrombocytes and spindle cells to platelets was recognized early and is generally accepted Gratia[33] obtained an incoagulable plasma by prolonged centrifugation, but did not believe plasma could be completely deprived of platelets

One of the important reasons for the confusion concerning the function of the platelet in coagulation has been the uncertainty as to the nature of the clotting factor which they liberate. It may be recalled that Alexander Schmidt at first believed that the fibrin ferment (thrombin) originated from the leukocytes Later he maintained that the leukocyte supplied a zymoplastic agent which activated the fibrin proferment which was dissolved in the plasma Morawitz,[87] Howell[58] and others postulated that the platelets supplied prothrombin together with thromboplastin Bordet and Delange[9] in contrast to the prevailing view contended that these cells furnished only cytozyme (thromboplastin) Although Tait and Green,[93] even as late as 1926, argued that the term prothrombin can be replaced by unruptured platelets, the concept of Bordet and Delange steadily gained A number of American workers, particularly Mills,[84] Eagle[19] and Ferguson[31] published results to show that the platelets did *not* contain prothrombin Interestingly, LeSourd and Pagniez[73] suggested that the action of the platelets might be enzymatic, but this was not too significant since many held that thromboplastin functioned as an enzyme

The cause of platelet agglutination, which may be considered a preliminary step in their participation in coagulation has been explained in various ways Wright and Minot[111] obtained evidence that agglutination or metamorphosis, as they termed it, was due to an agent in plasma which belonged to the globulin fraction of the plasma This agent significantly was inactivated at the same temperature at which prothrombin activity is destroyed and at which fibrinogen is precipitated They stated that the agent was not thrombin Mora-

witz and Brugsch[69] also considered the possibility of a specific agent that was responsible for the clumping of platelets. Roskam[82] postulated that platelets are covered with a film of protein as the preliminary step in agglutination. Apitz[4] and Lenggenhager[50] proposed that the layer was fibrin Subsequently it was shown, however, that agglutination occurs normally in the plasma from patients with afibrinogenemia [76] An observation of Fonio,[22] which has attracted little or no attention, may be so significant that it warrants being restated in his own words "We have in all our observations with native preparations seen how the addition of thrombin solution affects the thrombocytes causing an increased formation of pseudopods, assisting their attachment, and further how the formation of fibrin needles suddenly fixes them and brings about their disintegration " (Author's translation.)

**Clot Retraction.**—Hayem[30] in confirming the findings of Krauss[48] and Denys[17] that the platelets were reduced in purpura, further observed that blood in this condition failed to show clot retraction. The subsequent studies of LeSourd and Pagniez,[51] Bordet and Delange,[9] Fonio,[23] Opitz and Schober[72] and others established that retraction was quantitatively dependent on the number of platelets in the blood and that only intact platelets were functionally effective.

Curiously, a number of investigators including Achard and Aynaud,[1] Roskam,[83] Howell,[37] Hirose[34] and Pickering[75] maintained that clot retraction was not due to the platelets Even as late as 1931, MacKay[59] in his review article on platelets wrote "In retraction of the clot the platelet is of secondary importance and serves to augment the retractile property of the plasma " It seems fairly certain that the conclusions of these workers were based on experimental results that were technically faulty Their opinions were probably also influenced by the mechanical colloidal concept which had been introduced to explain clotting Clot retraction was considered comparable to the syneresis observed in a colloidal gel

Although few, if any, now question the fact that the platelet is the primary cause of clot retraction, the actual mechanism whereby it is accomplished remains an enigma LeSourd and Pagniez[52] in 1906 postulated *that retraction was brought about by a platelet ferment* or enzyme. Glanzmann[26] accepted this idea, named the enzmye

the knots of the fibrin net underwent shrinkage and in that manner caused a retraction secondarily of the whole clot A somewhat similar concept was developed by Tocantins[107] who showed by microscopic

study that intact platelets become adherent to the fibrin strands and in turn become the locus from which new needles of fibrin radiate In this process, twisting, bending and shortening of the fibers occurs and as a result the reticulum shrinks

The physiological significance of retraction has remained a matter of speculation. Frank[34] believed that it served as a means of closing injured vessels and wounds *Fonio*[33] developed a similar view and designated retraction as a physiological ligature This concept has been widely accepted by modern workers in spite of the fact that it is entirely speculative and is not supported by experimental evidence.

**Vasoconstriction Action of Platelets.**—It was observed early, both experimentally and clinically, that the intravenous injection of defibrinated blood or serum produced untoward effects which were not seen when non-clotted blood or citrated plasma was injected In 1884 Stevens and Lee[94] observed that a vasoconstricting substance appeared in blood following coagulation Two decades later the observation was again made by Brodie [11] These isolated observations probably would have been forgotten and lost if it had not been for the discovery and isolation of adrenaline which brought about a renewed interest in vasoconstricting agents present in the blood. O'Connor[71] (1911) in comparing the action of pure adrenaline with serum, concluded that the latter contained pressor agents that were not adrenaline but which resembled it. They appeared only after the blood had clotted Janeway and Park[41] almost immediately confirmed O'Connor's findings, and added the significant observation that the agent in serum acted directly on smooth muscle, and did not appear to have any relation to the sympathetic enervation of muscles. Its vasoconstriction action was brought about by a direct action on the muscle of the artery and not on the receptive substance of the neuromuscular junction In the same year Stewart (H A.) and Harvey[96] showed that this agent was not a protein, was thermostable and soluble in alcohol

To Stewart (G N ) and Zucker (T. F.)[95] belong the credit for the important discovery that the platelets are the source of the vasoconstrictor in serum Interestingly, this significant discovery is recorded in a short footnote to the main paper These authors were the first to recognize the possibility that this constricting action might have "an important part in the prompt sealing of wounded vessels in addition to the mechanical effect of the clot" It should be mentioned, however, that O'Connor earlier had observed that the sediment of citrated blood which was rich in platelets had a powerful effect on frog vessels At the time that these findings were reported, LeSourd and Pagniez[51] found that rabbit platelet extract when in-

witz and Brugsch[49] also considered the possibility of a specific agent that was responsible for the clumping of platelets. Roskam[82] postulated that platelets are covered with a film of protein as the preliminary step in agglutination. Apitz[4] and Lenggenhager[50] proposed that the layer was fibrin. Subsequently it was shown, however, that agglutination occurs normally in the plasma from patients with afibrinogenemia[76] An observation of Fonio,[22] which has attracted little or no attention, may be so significant that it warrants being restated in his own words: *"We have in all our observations with* native preparations seen how the addition of thrombin solution affects the thrombocytes causing an increased formation of pseudopods, assisting their attachment, and further how the formation of fibrin needles suddenly fixes them and brings about their disintegration." (Author's translation.)

**Clot Retraction.** – Hayem[30] in confirming the findings of Krauss[43] and Denys[17] that the platelets were reduced in purpura, further observed that blood in this condition failed to show clot retraction. The subsequent studies of LeSourd and Pagniez,[51] Bordet and Delange,[9] Fonio,[23] Opitz and Schober[72] and others established that retraction was quantitatively dependent on the number of platelets in the blood and that only intact platelets were functionally effective

Curiously, a number of investigators including Achard and Aynaud,[1] Roskam,[83] Howell,[37] Hirose[34] and Pickering[75] maintained that clot retraction was not due to the platelets Even as late as 1931, MacKay[59] in his review article on platelets wrote "In retraction of the clot the platelet is of secondary importance and serves to augment the retractile property of the plasma" It seems fairly certain that the conclusions of these workers were based on experimental results that were technically faulty Their opinions were probably also influenced by the mechanical colloidal concept which had been introduced to *explain clotting Clot retraction was considered comparable to the* syneresis observed in a colloidal gel

Although few, if any, now question the fact that the platelet is the primary cause of clot retraction, the actual mechanism whereby it is accomplished remains an enigma LeSourd and Pagniez[52] in 1906 *postulated that retraction* was brought about by a platelet *ferment* or enzyme Glanzmann[26] accepted this idea, named the enzmye retractozyme and postulated a disease entity, thrombasthenia, caused by hereditary deficient platelets Frank[24] offered a more mechanical explanation He believed that little clumps of platelets anchored in the knots of the fibrin net underwent shrinkage and in that manner caused a retraction secondarily of the whole clot A somewhat similar concept was developed by Tocantins[102] who showed by microscopic

physiological mechanism made up of a number of component parts

Hemostasis like every other scientific problem is rarely solved by any one individual or by the investigators of one laboratory or of one country. Only by the combined efforts of many workers from various nations is the final solution of the problem usually achieved. Unfortunately the European continent and England where much of the brilliant pioneer work on hemostasis was done has been scourged by two terrible wars with the result that many investigations that would have culminated in significant advances were rudely interrupted and often never resumed. In spite of all obstacles, the contributions to hemostasis have continued, and in recent years sufficient advances have been made to permit a correlation that should bring about a clarity and a rationality that heretofore has not been possible.

**Recapitulation.**—Knowledge concerning the physiology of hemostasis developed from studies in three different areas: vascular contraction after injury, coagulation of the blood, and platelets. Only after extensive advancements in these fields had been made, did it become apparent that effective hemostasis is not achieved by any one of these factors but only by an orderly correlation of all three forces.

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jected produced a drop in blood pressure. This principle likewise was heat stable and soluble in alcohol. A year later (1914) these French workers also confirmed the work of Stewart and Zucker. They expressed their perplexity in finding that platelet extract produced a systemic hypotension and a constricting action on a dog carotid artery ring in vitro Later Roskam[91] also observed the dual action of platelet extract and pointed out that it simulated histamine. Unfortunately, the problem has remained fallow, but the time seems propitious for restudying the relation of histamine to platelets

In a paper which is particularly valuable for its excellent review of the older literature, Janeway, Richardson and Park[42] in 1918 reported complete confirmation of the observations of O'Connor, Stewart and Zucker and others that platelets liberate a vasoconstrictor agent. The death of Janeway, and the disrupting influence of the First World War brought this research to a close. Its significance was underestimated so much that Macfarlane,[57] for instance, in his well-known article, "Critical Review The Mechanism of Haemostasis" written in 1941 failed even to mention this paper and the earlier work of Stewart and Zucker and of the other pioneer investigators

In reviewing broadly the historical development of the physiology of hemostasis one is struck with the periodic ascendency of interest in one phase of the mechanism and the setting aside of all other aspects of the general process Often the dominant objective was to prove that the particular mechanism under investigation was the primary or most important in hemostasis Thus, as recent as 1948 Macfarlane[58] wrote "that blood coagulation, which is after all a subsidiary and non-essential fragment of total bodily activity" But only 6 years earlier Apitz[3] stated that by various approaches it has been shown that "normal stanching is a function of blood coagulation and not dependent on the vascular response as has been the dominant view during the past few decades." (Author's translation ) Neither of these writers stressed the importance of platelets, but interest in these cells as factors in hemostasis had its peak during the era of Bizzozero and Hayem, and again from 1911 to 1918 when their vasoconstriction action was investigated But after this short period, interest in platelets lapsed and an increasing indifference to these cells became apparent In 1931 MacKay[59] in his article, "The Blood—Platelet: Its Clinical Significance" made the following statements "It is doubtful if the platelet factor in the clotting process is of more than secondary importance" Tocantins[100] in 1947 writing on the [illegible] of hemostasis fails even to mention the [illegible]

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**Function of Platelets in Coagulation.**—The first issue of basic importance to be decided is whether the platelet is essential and *indispensable for coagulation. Can clotting occur in the complete* absence of platelets? Due to the technical difficulties of removing platelets from plasma completely and of avoiding the contamination of the blood with a trace of tissue thromboplastin during the collection, it becomes a formidable task to obtain a plasma sufficiently platelet-free to become incoagulable. Patton, Ware and Seegers[13] have succeeded by meticulous care in collecting the blood and by high centrifugation in obtaining a plasma which remained fluid for more than three days at room temperature. They employed silicone-coated apparatus, following the work of Jacques and his associates[11] who discovered that by means of the reagent, silicone, a surface can be prepared which like healthy endothelium does not allow platelets to adhere and consequently preserves them. Patton and his associates were justified in concluding that no evidence can be found "to support the view that plasma may contain a soluble factor which can initiate clotting independent of platelet action."

When a minute amount of thromboplastin is added to platelet-free plasma, coagulation begins promptly. *A priori* one would conclude that platelets on rupturing yield thromboplastin, but Quick[23] found that platelets contain little if any thromboplastin and this has been confirmed by Ware, Fahey and Seegers.[19] Since the platelets do not contain thromboplastin and yet the coagulation defect resulting from platelet deficiency can be corrected by the addition of thromboplastin, it appears logical to conclude that platelets while not supplying thromboplastin directly, are nevertheless responsible for making this clotting factor available. Quick[23] postulated that thromboplastin exists in plasma as the inactive precursor, thromboplastinogen, and that platelets on rupturing furnish the activator.

Further confirmatory evidence for this view is obtainable from hemophilic studies. The coagulation defect in this disease can be promptly corrected by the addition of thromboplastin. Since the platelets in hemophilic blood are unusually stable, it has been assumed by some investigators that the defect was localized in these cells. But Pickering[19] found that the addition of saponin to hemophilic blood, which caused complete disintegration of platelets, did not restore normal coagulation. Recently Quick[23] reported that the addition of hemophilic platelets to platelet-poor normal plasma brought about normal clotting. On the basis of these findings, it appears that hemophilic blood is deficient in the precursor, thromboplastinogen, and that the fault is not in the platelet activator.

By means of a new quantitative procedure, the prothrombin con-

## Chapter 2

# A NEW THEORY OF THE COAGULATION OF THE BLOOD AND A UNIFIED CONCEPT OF HEMOSTASIS

No one who has witnessed uncontrollable bleeding in hemophilia, in congenital hypoprothrombinemia or in severe Dicumarol poisoning will question the importance of the coagulation reaction in hemostasis In fact, it seems quite certain that the clotting mechanism occupies a central position in the physiological process of stanching, and that no comprehensive concept of the latter can be formulated unless a logical and adequate explanation of coagulation can be offered.

In the past the best guide was the classical theory, but it had inherent weaknesses and deficiencies which necessitated its replacement by a new and more comprehensive hypothesis The principal shortcomings of the theory were.

1. It failed to define the function of the platelets.
2. It had no explicit explanation for the source of thromboplastin
3. It considered the action of thromboplastin and calcium to be catalytic
4. It assumed prothrombin to be a unit substance
5. It offered no exposition for the autocatalytic nature of the coagulation reaction
6. It had no adequate explanation for the maintenance of the fluidity of the blood
7. It did not correlate the coagulation reaction with the generalized mechanism of hemostasis

During the past few years prodigious progress has been made in the study of coagulation As a result it is possible to formulate a new concept, which should successfully supplant the classical theory and meet the shortcomings of the older theory In attempting to present this simple workable hypothesis of coagulation, caution was taken to avoid the maze of conflicting theories and an attempt was made to correlate as well as integrate the pertinent findings which rest on sound observations and well-controlled experimental evidence

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for the labilization and lysis of platelets. Fonio[9] and more recently Zatti[11] have found by direct microscopic observation that thrombin acts on platelets causing their agglutination and lysis

It appears certain, therefore, that thrombin is essential for platelet lysis and that a rough surface is not the primary factor. Since plate-

the wettable surface and that a platelet coming in contact with this layer immediately becomes sticky and unstable A surface such as silicone lacking the power to adsorb thrombin has no attraction for platelets

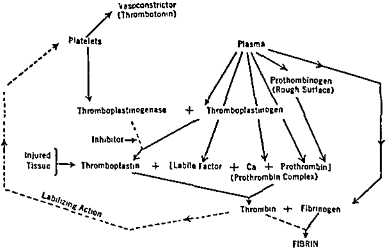



FIG 2 —The coagulation mechanism The labilizing action of thrombin brings about an autocatalytic or chain reaction which may be designated the thrombinogenic cycle The removal of thrombin through absorption by fibrin constitutes the most important means for controlling the chain reaction

The acceptance of the finding that thrombin labilizes platelets, permits the formulation of a logical explanation for the autocatalytic reaction of coagulation As soon as a small amount of thrombin is formed, platelets begin to be labilized and to disintegrate This immediately leads to an activation of thromboplastinogen, which in turn causes prothrombin to be converted to thrombin The new thrombin labilizes more platelets and an accelerated reaction results.

sumption test, which measures the amount of prothrombin remaining after blood has been allowed to coagulate under standardized conditions, Quick[23] has been able further to substantiate that the essential rôle of platelets in the coagulation of blood is the activation of thromboplastinogen He found that when platelets were removed by high centrifugation, the resulting plasma showed little diminution of prothrombin after complete coagulation of the fibrinogen even when the clotted plasma was allowed to stand 30 minutes or more. Later, he with his associates[30] showed that the speed of prothrombin activation was a function of the number of platelets and the type of curve obtained was typical of an enzymatic reaction (Fig. 1) There is, there-

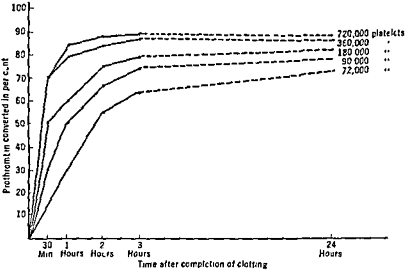



FIG. 1 —The influence of the number of platelets on the speed of prothrombin conversion

fore, experimental evidence that the platelets are responsible for making thromboplastin available especially on the basis of data obtained with the prothrombin consumption test which measures the amount of thromboplastin reacting with prothrombin In hemophilia little prothrombin becomes activated during coagulation because there is, according to Quick, a congenital lack of thromboplastinogen Even though the platelets are normal, they cannot produce thromboplastin because the precursor is lacking

**The Labilization of Platelets.**—The significant observation of Burker[5] that the conditions or factors which prevent clotting also inhibit platelet disintegration, is an important key to the study of coagulation Platelets remain intact in decalcified, in heparinized,

method. Seegers,[31] as well as Fantl[8] and Owren,[17] considered the new factor an accelerator, whereas Quick claimed that this agent reacts stoechiometrically in the reaction.

Quick and Stefanini[33] in a recent study were able to prove that the labile factor did not accelerate the conversion of prothrombin to thrombin. They found that the prothrombin time was not shortened by adding a large excess of labile factor to human oxalated plasma. In an extension of this investigation[34] they were able to trace the source of confusion, which undoubtedly misled others to conclude that the labile factor was an accelerator They found that when normal oxalated human plasma is stored in glass, the prothrombin time as determined by the one-stage procedure steadily increases, but when a small quantity of deprothrombinized rabbit plasma which is rich in labile factor is added, the mixture will have a prothrombin time as low as 8 seconds which is shorter than that of fresh plasma which is *12 seconds If the plasma is stored in silicone-coated tubes, it like*wise will progressively lose prothrombin activity on storage, but when excess labile factor in the form of deprothrombinized rabbit plasma is added, the prothrombin time is restored to the original 12 seconds. They further found that when hemophilic plasma is placed in a glass tube and allowed to stand, at 37° C. the prothrombin time which was 12 seconds gradually decreases to approximately 8 seconds in the course of an hour. If the plasma is placed in a silicone-coated tube and thrombin added to induce coagulation, the prothrombin time remains 12 seconds

A satisfactory explanation for these results demands a radical departure from the prevailing concept of prothrombin The increase

that plasma contains prothrombin in a precursor state in addition to the active prothrombin For the conversion of the precursor, a rough surface such as glass is required. It is likely that other factors play a rôle in this reaction, but further study will be required before a satisfactory explanation of this activation can be attempted

It is very probable that failure to recognize the possibility of the existence of an inactive form of prothrombin, has been responsible for erroneously postulating the participation of activators in the conversion of prothrombin to thrombin Interestingly Bordet[2] maintained that all of the prothrombin or serozyme existed in the inactive state, but his theory received very little attention While present studies indicate that only part of the prothrombin in human blood is inactive, the basic idea of Bordet that a precursor (a proserozyme) exists is

This chain reaction can be designated the thrombinogenic cycle Its position in the coagulation mechanism can readily be recognized by studying figure 2.

Normal blood contains no free or active thromboplastin. It can only be produced by the action of a platelet factor on the precursor. A deficiency of thromboplastin can result from a lack of thromboplastinogen, which occurs in hemophilia; from a lack of platelet activator which may be the result of either a qualitative defect of the platelet or a quantitative deficiency, *i e.*, thrombocytopenia; or from an inhibitor which antagonizes the platelet activator A deficiency of thromboplastin can readily be determined by the prothrombin consumption test [21]

**The Prothrombin Complex.**—The majority of investigators have accepted from the time of Pekelharing and Schmidt that thrombin is formed from a precursor, prothrombin, which was considered a discrete compound In 1943 Quick[22] found that when he mixed stored oxalated human plasma in which the prothrombin activity had been markedly reduced, with an equal volume of hypoprothrombinemic plasma from a Dicumarolized animal, the resulting mixture had a prothrombin potency even greater than normal. He concluded that the prothrombin consisted of two distinct factors, one of which was destroyed by storage, the other diminished by Dicumarol. He further noted that aluminum hydroxide adsorbed little of this new factor. The following year, Owren[16] in Oslo studied a patient with a hypoprothrombinemia whose plasma was made normal by the addition of normal plasma deprothrombinized by an adsorbent. He concluded that this patient did not lack prothrombin, but a new clotting principle which he named Factor V It is apparent that the latter is identical with the component discovered a year earlier by Quick Because of the war, Owren's work did not become known until 1947 At this time a number of other investigators, particularly Fantl,[7] Seegers and their associates[40] described what they considered were new clotting factors and which they designated as accelerators. Further study, however, showed that these new agents were the same as Quick's, which he originally designated Component A[22] but later renamed labile factor [27]

In his original study Quick[22] found that the concentration of the labile factor was much higher in rabbit and dog plasma than in human plasma, and later he with Stefanini[35] determined that rabbit plasma contained 50 times and dog plasma 10 times as much labile factor as human plasma. Murphy and Seegers[15] confirmed in part this finding, and they postulated that the new factor (Ac-globulin) markedly influences the determination of prothrombin activity by the one-stage

**The Thrombinogenic Cycle and Its Control.**—For the formation of thrombin, thromboplastin and the various components of the prothrombin complex must be present in adequate amounts. For the cycle to function, the thrombin must labilize platelets which in turn yield the agent that activates thromboplastinogen As each cycle is completed more thrombin is formed, and this results in a marked acceleration of clotting since the speed of clotting is almost solely a function of the concentration of thrombin

Thrombin is an enzyme and the amount formed even from as little as 1 cc of blood is enough to coagulate potentially all the fibrinogen of the circulating blood. It is obvious, therefore, that the production as well as the action of thrombin must be controlled. The existence of an antithrombin in normal blood has been recognized since the early studies were made on thrombin Lenggenhager[17] and Quick[21] found that the active neutralizer of thrombin was brought about by a constituent present in the albumin fraction. This natural antithrombin, significantly does not prevent the coagulation of fibrinogen Only after the latter is completely converted to fibrin does the antithrombin remove the active thrombin Thus, if one adds a minute amount of thrombin to highly centrifuged hemophilic plasma, repeated crops of fibrin can be removed for as long as 5 days, which shows the ineffectiveness of the natural antithrombin

Since the activity of thrombin obviously cannot be controlled by the natural antithrombin, also known as Albumin X, some other physiological scheme must be sought to account for the controlling and the localizing of the action of thrombin Quite accidentally Quick and Favre-Gilly[29] made an observation which clearly gave the key to the mechanism whereby the action of thrombin is controlled These investigators found that in normal coagulation of blood only a trace of prothrombin was converted to thrombin, and that it was only after the serum was separated from the clot that a rapid disappearance of prothrombin occurred It thus became evident that the fibrin reticulum with its tremendous surface adsorbs thrombin so effectively that little is available for acting on the platelets The chain reaction thus becomes slowed and is put under control

The observation that only a minute amount of prothrombin is utilized for forming the clot, challenges the widely accepted concept of the rôle coagulation plays in hemostasis which is that a fibrin clot is promptly produced and acts as a mechanical plug Ever since Petit called the thrombus within a vessel a bouchon and the one outside the vessel, a couvercle, nearly everyone has tacitly accepted the rôle of the clot as a mechanical stopper or seal In regard to hemostasis, the vascular system has been viewed from a plumber's point of

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## Chapter 3

# CLASSIFICATION OF THE HEMORRHAGIC DISEASES AND THEIR DIAGNOSIS

SINCE knowledge concerning even the common hemorrhagic diseases is still incomplete, any scheme of classification must be considered tentative and temporary—not permanent or final. This does not gainsay the value of a classification, for without it both diagnosis and treatment become more difficult. It is with this purpose that the following classification is offered:

### Classification of the Hemorrhagic Diseases

I Bleeding diseases due to vascular defect (Angiostaxis)
- A. Anatomical defect
  - *a*. Scurvy
  - *b* Hereditary telangiectasia
- B Physiological Dysfunction
  - *a* Hereditary pseudo-hemophilia
  - *b*. Purpuras (Without thrombocytopenia)

II Bleeding diseases due to factors in the blood (Hematostaxis)
- A. Hypothromboplastinemias
  - *a* Lack of platelet activity.
    - 1 Thrombocytopenia
    - 2 Qualitative platelet defect
    - 3 Platelet enzyme inhibitor
  - *b* Deficiency of thromboplastinogen.
    - 1 Hemophilia.
    - 2 Acquired hemophilia-like disease (Tentative)
- B Hypoprothrombinemia (Decreased prothrombin activity)
  - *a* Congenital
    - 1 Deficiency of labile factor.
    - 2 Deficiency of free prothrombin (Hereditary)
    - 3. Deficiency of free and total prothrombin (Familial)
  - *b* Acquired
    - 1 Dietary deficiency
      - i Hemorrhagic disease of the newborn
        Sterilization of intestinal tract

ii. Faulty absorption.
Obstructive jaundice
Biliary fistula.
Sprue.

2. Liver damage
    i Atrophic cirrhosis.
    ii. Hepatitis
3 Toxins
    i Dicumarol.
    ii. Salicylate.
    iii Penicillin.

C. Hypofibrinogenemia
   *a* Congenital
   *b* Acquired

D Platelets deficient in vasoconstrictor principle (Hypothetical)

E Heparinemia.

## ANGIOSTAXIS

Only two well-known hemorrhagic diseases can be unequivocally ascribed as being due to a vascular defect They are scurvy and hereditary telangiectasia

**Scurvy.**—In this disease, the small blood vessels become impaired from the lack of the intercellular cementing substance, collagen The diagnosis offers little difficulty In the adult, the dietary history alone is often enough to make the diagnosis The bleeding spongy gums, the characteristic perifollicular petechia-like eruption especially on the legs, the asthenia with dyspnea and the deep muscle and joint pain present a combination found in no other disease In the infant, the extreme tenderness of the legs, the spongy gums, swellings near and about the large joints and the bleeding tendency should immediately suggest the diagnosis The striking and prompt response to a diet high in vitamin C establishes the diagnosis The tourniquet test is positive. Other hemostatic tests including the bleeding time are normal.

**Hereditary Telangiectasia.**—This disease is caused by an inherited malformation of the capillaries of the skin and of the mucous membrane. The capillary defect manifests itself as localized dilatations resulting in minute angiomas often spider-like in appearance which have a tendency to bleed when injured Although the lesions may make their appearance in early youth or even in childhood, the

TABLE 1.—DIFFERENTIAL DIAGNOSIS OF COMMON HEMORRHAGIC DISEASES WITH A NORMAL PROTHROMBIN TIME

| | *Hemophilia* | *Pseudo-hemophilia* | *PURPURA* Non-thrombo-cytopenia | *PURPURA* Thrombocytopenia | *Telangiectasia* |
|---|---|---|---|---|---|
| Heredity | Recessive sex-linked | Dominant | | | Dominant |
| Sex affected | Male | Male and female | | | Male and female |
| Sex transmitting active disease | Female | Male and female | | | Male and female |
| Bleeding | | | | | |
| Cause | Usually trauma | Trauma | Spontaneous | Spontaneous | Usually trauma |
| Onset after injury | Delayed | Immediate | Immediate | Immediate | Immediate |
| Petechial | Absent | Rare | Fairly common | Common | Absent |
| Mucosal | Uncommon | Common | Common | Common | Common |
| Spontaneous Epistaxis | Occasional | Common | Rare | Common | Common |
| Deep hemorrhage and hematomas | Common | Infrequent | Absent | Absent | Absent |
| Hemarthrosis | Common | Fairly infrequent | Absent | Absent | Absent |
| Laboratory finding | | | | | |
| Coagulation time | Prolonged | Normal | Normal | Normal | Normal |
| Bleeding time | Normal | Prolonged | Normal | Prolonged | Normal |
| Prothrombin consumption | Decreased | | Normal | Decreased | |
| Platelet count | Normal | Normal | Normal | Decreased | Normal |
| Clot retraction | Normal | Normal | Normal | Absent | Normal |
| Tourniquet test | Negative | Usually normal | Positive | Positive | Normal |

ii. Faulty absorption.
  Obstructive jaundice.
  Biliary fistula.
  Sprue.
2. Liver damage.
  i Atrophic cirrhosis.
  ii Hepatitis
3 Toxins
  *i Dicumarol.*
  ii Salicylate
  iii. Penicillin

C Hypofibrinogenemia.
  *a* Congenital
  *b* Acquired

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fairly certain that the defect manifests itself in the primary response to injury which may be either the reflex contraction or lack of stickiness of endothelium when irritated. The platelets and the coagulation mechanism appear to be entirely normal. On the basis of one case studied by Reid,[47] the vasoconstricting activity of platelets can be considered to be normal. One can conclude that the abnormality resides in the vessel itself. It is not an anatomical, but a physiological deficiency. Whether only the capillaries are affected or whether the arterioles also are involved has not been determined.

Oddly enough, the bleeding time which is the only available means to study this condition shows far greater variations than can be attributed to mechanical factors or faulty technique At times the bleeding time may be within normal limits and at other times may be prolonged to one hour or more

The diagnosis in the typical case is extremely simple The hereditary history, the prolonged bleeding time, the absence of telangiectasia, and normal coagulation tests establish the diagnosis. Unfortunately, the primary disease can be complicated by a superimposed thrombocytopenia, as the writer recently encountered, and perhaps by other conditions. Clinically the bleeding may simulate that of purpura or of hemophilia Epistaxis is frequent Easy bruising is also common Bleeding after dental extractions, cuts and minor injuries are seen often Hemarthrosis which is not found in purpura, has been reported in a number of patients with pseudohemophilia. For a detailed account of the disease, the monograph of Quick[17] and the review of Estren, Medal and Dameshek[14] should be consulted

An unequivocal diagnosis of hereditary pseudohemophilia can only be made if a history of heredity can be established, and a prolonged bleeding time is found in the presence of normal clotting factors, including platelets It is probable that an acquired type of pseudohemophilia may exist Carpenter and Allen,[6] and Estren and his associates[14] have each reported a case that fits into that category In each the bleeding tendency developed fairly suddenly during the third decade of life and in neither could a family history of bleeding be found

Attention should be called to the fact that a positive family history of bleeding can usually be established more easily in telangiectasia and in pseudohemophilia than in hemophilia In the first two diseases, the inherited characteristic is dominant and occurs in both sexes, whereas in hemophilia it is a sex-linked recessive and the disease may be transmitted for several generations occultly through females

**Purpura.**—Although the term purpura is often used loosely to describe all types of hemorrhages into and below the skin, the disease

full development of the disease usually occurs during the fourth decade of life and the bleeding tendency increases with age.

The diagnosis rarely offers any difficulty. The lesions are readily recognized and occur most frequently in the nose, mouth, neck, face and finger tips The most common form of hemorrhage is epistaxis The bleeding can often be traced to visible telangiectatic lesions The disease is hereditary, is transmitted as a non-sex linked dominant, and occurs in both males and females Occasionally no hereditary history is obtainable This may be due to atavistic skipping, or to such extreme mildness of the disease in a transmitter that it fails to give clinical evidence of its existence

While the disease is easily differentiated from the well-known bleeding diatheses, recognition of hereditary telangiectasia as the cause of certain forms of hemorrhage that might easily be considered secondary to other diseases may at times tax clinical acumen. Thus, the hemoptysis resulting from telangiectatic lesions of the pulmonary tree may easily be mistaken for tuberculosis Likewise gastric and intestinal bleeding traceable to traumatized angiomata might be erroneously attributed to ulcer, cancer, and other conditions.

The laboratory tests are of little value in diagnosis, for all are normal in uncomplicated telangiectasia In table 1 a comparison of the chief characteristics of this disease with other hemorrhagic diatheses is given

**Hereditary Pseudohemophilia.**—This hemorrhagic disease is characterized by a prolonged bleeding time and a normal coagulation mechanism. It is inherited as a simple dominant, occurs in and is transmitted by both sexes While the term pseudohemophilia is not entirely satisfactory, it has been used extensively by many writers including von Willebrand,[52] who was the first to recognize the condition as a clinical entity The term hereditary athrombocytopenic purpura which has been proposed is unsuitable, for not all of the patients have purpura, and petechiæ are very uncommon The condition should not be confused with Glanzmann's[17] hereditary hemorrhagic thrombasthenia, which is still an ill-defined condition It is erroneous to consider the disease identical with thrombasthenia since the existing evidence fails to show any platelet dysfunction in pseudohemophilia

The basic defect in this disease which causes the bleeding tendency remains undetermined. While it has been found that in certain cases the capillary loops have bizarre shapes,[25] others[32] have reported normal capillaries as observed in the nail bed microscopically It is generally agreed that the prompt contraction that is observed when normal capillaries are injured, is lacking in this disease It seems

capillary tone or by the production of a dilator in excessive amounts.

Many elaborate classifications of purpura have been proposed, but they have not greatly advanced the study of this group of diseases Often in such classifications, entities like senile purpura are included. If this condition is due to degenerative changes of the small blood vessels as some believe, it is not a true purpura. It seems desirable to limit the term purpura to capillary hyperpermeability due to an extrinsic factor and to exclude hemorrhagic conditions which arise from an intrinsic anatomical defect

It is reasonable to suspect that a close relation exists between the thrombocytopenic purpura and the purpuras with a normal platelet count. It is difficult to understand why the well-known anaphylactoid types of purpura known as the Schonlein-Henoch Syndrome have a consistently normal platelet count

The diagnosis of purpura without thrombocytopenia is made mainly by exclusion. The presence of a red macular exanthem which persists on pressure and comes in recurrent crops suggests purpura.

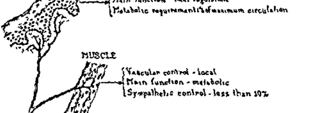



FIG 3 — The control of circulation in the skin and muscles

The appearance of small petechiæ and ecchymoses make the diagnosis more certain Purpura must be differentiated from pseudohemophilia and telangiectasia This is rather simple since the bleeding time in non thrombocytopenic purpura is usually normal and no telangiectasias are detectable In purpura, the routine tests for coagulation and hemostasis are normal with the exception of the tourniquet test which often is positive Since purpura is only one of a characteristic group of symptoms in the Schonlein-Henoch Syndrome, the diagnosis must be made on the basis of the whole symptom complex

## HEMATOSTAXIS

A practical classification of the hemorrhagic diseases arising from defects of the coagulation mechanism became possible through a series

entity designated purpura in this treatise is defined as a diapedetic bleeding into skin causing petechiæ and ecchymoses and into mucous membrane giving rise to spontaneous hemorrhage. The defect is solely in the capillaries and the recent studies of Humble[19] indicate that purpuric manifestation arises only at the arteriolar end of the capillary loop Humble points out that this arteriolar-capillary junction is of great importance in the maintenance of blood flow and suggests that it may perhaps be subject to selective poisoning. At this point in the vascular system, the blood pressure is still relatively high, but the protective muscular coat is no longer present, which explains its high vulnerability.

Since purpura can and often does occur with no diminution of platelets, it can be concluded that these cells are only indirectly related to purpura Likewise the coagulation mechanism has no direct relation to this bleeding condition

*It is significant that the capillaries of the skin and mucous membrane* are mainly involved, while there is no evidence that the capillaries supplying muscle tissue are affected. Perhaps this observation may serve as a key to a better understanding of the cause of purpura. According to Krogh,[22] the capillaries supplying muscles are mostly collapsed when the tissue is in the resting stage On stimulating the muscle, the blood flow increases and the capillaries open and dilate The stimulus comes from the accumulation of metabolites which have a dilating effect Barcroft and his associates[5] have reported findings which indicate that less than 10 per cent of the blood flow through skeletal muscles is under the control of the sympathetic nervous system Since the demands for increased blood supply is local, it is logical to expect that the control likewise is local and not systemic or mediated through the central nervous system

The capillaries of the skin, in contrast, present a different situation. Not more than 1 per cent of the maximum potential blood flow is required to meet the metabolic needs of the skin The important function of the circulation of blood through the skin is to regulate body temperature and to dissipate heat This necessitates that the control be central The circulation of the blood in the skin is, therefore, not regulated locally, but systemically In figure 3 the capillary blood flow through muscles and skin is presented based on data taken from a study of Goetz [18]

It is probable that the blood flow through the capillaries of the skin is, as Humble[19] suggests, regulated at the capillary-arteriolar junction. Whether the control is nervous or hormonal is not known, but one may postulate that the abnormal increase in capillary permeability is brought about either by the lack of a factor essential for

capillary tone or by the production of a dilator in excessive amounts.

Many elaborate classifications of purpura have been proposed, but they have not greatly advanced the study of this group of diseases Often in such classifications, entities like senile purpura are included. If this condition is due to degenerative changes of the small blood vessels as some believe, it is not a true purpura It seems desirable to limit the term purpura to capillary hyperpermeability due to an extrinsic factor and to exclude hemorrhagic conditions which arise from an intrinsic anatomical defect.

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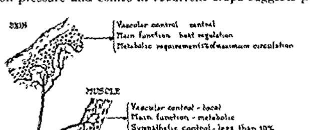



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A practical classification of the hemorrhagic diseases arising from defects of the coagulation mechanism became possible through a series

of basic studies initiated in 1934 which yielded simple procedures for determining quantitatively prothrombin and recently prothrombin consumption. Before these tests became available, chief reliance had to be placed on the coagulation time, which unfortunately gave misleading results in a number of important bleeding conditions It failed for instance to disclose a clotting defect in thrombocytopenic purpura and even in moderately severe hypoprothrombinemia. The time has come to recognize that the coagulation test, no matter how meticulously refined, remains an empiric procedure which is entirely unphysiological. While a prolonged coagulation time is always significant, a normal finding is meaningless, since a serious coagulation defect may exist when the coagulation time is normal or even shorter than normal

The term, "coagulation defect," is unsatisfactory since it is merely a measure of the time required for sufficient fibrin to form to produce a rigid clot. In such a clot less than one-third of the fibrinogen may be converted to fibrin, and less than 1 per cent of the prothrombin may be activated The term, thrombinogenesis, is much more meaningful since the production of thrombin is indispensable for hemostasis while fibrin is not It is suggested, therefore, that defective thrombinogenesis rather than defective clotting be used to designate the conditions in which the production of thrombin is decreased and that the term, hematostaxis, cover all the bleeding diseases in which the fault resides in the blood

**Hypothromboplastinemias.**—According to the theory of coagulation presented in figure 2, the production of thrombin depends on a group of factors comprising the prothrombin complex, and on thromboplastin, which is derived from a plasma precursor, thromboplastinogen, through the activation of a platelet factor A deficiency of thromboplastin can arise from (1) lack of platelets, (2) an inhibitor of the platelet activator, or (3) a deficiency of thromboplastinogen. A deficiency of any one of these factors will result in a markedly incomplete activation of prothrombin, which can readily be measured by the prothrombin consumption test. This procedure establishes the existence of hypothromboplastinemia just as the prothrombin *time test* indicates hypoprothrombinemia Neither test specifically determines any one of the factors responsible, but other methods have been developed to serve this purpose

**Thrombocytopenia.**—In the discussion of purpura, it was pointed out that a purpuric type of a hemorrhagic diathesis can occur with a normal platelet count It is well known, moreover, that an exceedingly and persistent low platelet count may be unaccompanied by hemorrhage. It is reasonable to conclude that the capillary hyper-

permeability observed in purpuric hemorrhage is not caused by lack of a platelet factor. Nevertheless, the severity of the hemorrhagic tendency is usually much greater in thrombocytopenic purpura than in the non-thrombocytopenic types. Apparently bleeding does not occur until capillary dysfunction has been produced, but when established the bleeding is accentuated by a decrease in platelets.

To understand better the possible effect of thrombocytopenia it should be recalled that platelets have at least two clearly defined functions; namely, to activate thromboplastinogen and to supply a vasoconstricting principle Since the agent that converts thromboplastinogen to thromboplastin appears to be an enzyme, it is obvious that a small number of platelets may still supply enough activator to prevent as serious a deficiency of thromboplastin as occurs in hemophilia It should also be remarked that Doan[13] has recently obtained evidence that the platelet count in the peripheral blood may not be a true measure of platelet production There is apparently a high consumption of platelets for normal physiological processes, and the platelets in the blood appear to represent the surplus. The platelet count, therefore, may not always be a true criterion of thrombocytopenia

For a critical study of thrombocytopenia, the bone marrow must be examined, and by this means two general types can be differentiated In the first the megakaryocytes are normal or hyperplastic, while in the second the marrow is aplastic and the megakaryocytes often greatly reduced and non-functioning The second type is encountered in aplastic anemia and is produced by certain poisons of which benzene is the most notorious The bleeding tendency is severe in this form of thrombocytopenia

Thrombocytopenia with relatively normal bone marrow is the more common Under this class can be grouped the primary or idiopathic, commonly known as Werlhof's disease and the various secondary purpuras In these the decrease in platelets appears to be due to excessive destruction. In Werlhof's disease the spleen plays such an important rôle in this destruction, that splenectomy cures, at least clinically, the disease. The same is also true in panhypersplenism

There is much experimental evidence to support the view that the first step in the production of thrombocytopenia is agglutination of platelets It follows logically that the factor that produces this agglutination is likely to be the causative agent of the thrombocytopenia It has been postulated that a specific compound is responsible for this agglutination Some have maintained that the substance is heparin,[10] but this has been disproved [14, 41] In a recent study on the pathogenesis of the thrombocytopenia resulting from Sedormid (allyl-

isopropyl-acetyl-carbamide) Ackroyd[1] has obtained results which are most provocative He found that when he added the drug to the blood of a patient sensitized to Sedormid, agglutination of platelets occurred in vitro, thus indicating that the platelets themselves become sensitized In a later study, Ackroyd[2] found that the offending drug did not act directly on the platelets, but that it apparently reacted with a substance in the plasma of the sensitized patient and thereby produced an agent which agglutinated the platelets He further *noted that the drug inhibited clot retraction.* Such findings as reported by Ackroyd, may be perhaps the turning point in the approach to the problem of the secondary thrombocytopenic purpura.

An acute type of thrombocytopenic purpura with generalized platelet thrombosis may occur. Twelve such cases have been described since 1925 Singer and his associates[49] have summarized these cases and have presented a critical discussion of this clinical entity. *A recent editorial in Annals of Internal Medicine*[13a] *also thoroughly reviews* and discusses this disease A significant new idea concerning the cause of this clinical entity has been offered by Meacham and his co-workers [35a] They question whether the platelet emboli constitute the primary occlusive lesion *They believed that the primary* cause is a degeneration of the arteriolar wall which is responsible for the massing of platelets in the affected area

While the spleen and the bone marrow are the two organs that should always receive first consideration in thrombocytopenic purpura, one should not overlook the fact that other organs may at least indirectly bring about purpura and thrombocytopenia Whitesell and Snell[43] have pointed out that a fall in platelets as well as an increased capillary permeability are common in parenchymal diseases of the liver. It becomes imperative, therefore, not to make a diagnosis of idiopathic or primary thrombocytopenic purpura until liver disease as a probable *cause has been excluded*

The diagnosis of thrombocytopenia depends first of all on the platelet count The prothrombin consumption test is an additional valuable confirmatory method Bone marrow examination is necessary to determine whether the decrease in platelets results from excessive destruction or decreased production.

A qualitative defect of platelets although theoretically probable, has remained uncertain Whether a clinical entity such as Glanzmann's hereditary hemorrhagic thrombasthenia in which only clot retraction is impaired or absent actually exists, is open to question Clot retraction is too ill-defined a phenomenon to be the sole means for establishing a hemorrhagic diathesis. With the development of

the prothrombin consumption test, a new means has become available to test the thrombinogenic activity of platelets, and it is hoped that in addition a simple procedure can be perfected to test the vasoconstricting property of these cells.

**Platelet Enzyme Inhibitor.**—When normal blood or plasma is added to hemophilic blood, the coagulation time is reduced to normal, or often to a value below that of the blood added. This can be considered fairly satisfactory proof that the coagulation defect in true hemophilia is not due to an inhibitory agent. In 1940 Lozner and his associates[14] encountered a bleeding diathesis in a 61 year old Negro which closely resembled hemophilia. The negative family history, and the occurrence of the disease late in life made the diagnosis of hemophilia improbable. They concluded that the prolonged coagulation time was brought about by a circulating anticoagulant since addition of the patient's plasma to normal blood caused a delay in coagulation. A year later Lawrence and Johnson[23] studied a hemophiliac who likewise had in his blood a similar anticoagulant. Munro and Jones[40] also reported such an agent appearing in the blood of a hemophiliac as the result of numerous transfusions. In these patients a transfusion with normal blood neither reduced the coagulation time nor brought about clinical improvement

Fantl and Nance[15] reported a similar acquired condition in a 34 year old woman It is significant that the case of a hemophilic-like disease in a woman reported by Joules and Macfarlane[21] earlier did not show such an anticoagulant substance in her blood

Madison and Quick,[28] and Chargaff and West[7] have each reported an additional case of a bleeding disease in a woman closely simulating hemophilia, but in neither report was the study complete enough to exclude the presence of an anticoagulant Even from this limited number of cases, one is justified to conclude that an acquired hemophilic-like disease can occur in a woman. In some the causative factor is a circulating anticoagulant while in others it appears probable that the condition is similar to hemophilia and is independent of any inhibitory factor

Studies particularly by Munro,[27] Craddock and Lawrence[11] as well as others[40] show clearly that this new anticoagulant is not heparin, not an antithrombin, antiprothrombin, or an antithromboplastin. Munro[27] found it was in the globulin fraction and later he[33] as well as Craddock and Lawrence[11] traced it further to the gamma globulin. The latter workers postulated that it inhibited the antihemophilic globulin Quick and Stefanini,[45] who studied one case corroborated the findings that the anticoagulant did not correspond to any of the

known inhibitors. The same conclusion had been reached by Dieter, Spooner and Pohle,[12] who had studied the case earlier. The blood of this patient when added to normal blood, not only increased the coagulation time but also strikingly decreased the prothrombin consumption. Quick and Stefanini postulated that this anticoagulant inhibits the platelet activator and that this brings about a deficiency of thromboplastin since the thromboplastinogen cannot be converted to the active form Craddock and Lawrence[11] have proposed the interesting hypothesis that this anticoagulant is an antibody resulting from the injection of whole blood, plasma or Fraction I of Cohn Conley and his associates,[9] however, have found this inhibitor in non-hemophilic patients who had received no transfusions.

On the basis of these findings one can conclude that an anticoagulant which is perhaps a gamma globulin and which inactivates the platelet enzyme can arise either as the result of transfusions (particularly from the injection of the antihemophilic fraction of Cohn) or secondary to a number of systemic diseases (in the case of Quick and Stefanini, it was pemphigus) The inhibitor can occur in either a non-hemophilic subject or in a true hemophiliac

The diagnosis of this bleeding diathesis is readily made Blood is drawn from the patient and from a normal subject using silicone-coated glassware The coagulation time by the Lee-White method and the prothrombin consumption time are done on both bloods and on an equal mixture of the two bloods. In uncomplicated hemophilia, the coagulation time of the mixture is normal, whereas it is prolonged if the blood contains the inhibitor A more quantitative method has been developed by Conley *et al* [9] In their procedure, the pathological blood is highly centrifuged to obtain a platelet-free plasma which is added in increasing amounts to 1 cc samples of normal blood and the effect on the clotting time is determined

**Hemophilia** —This disease is caused by an inherited deficiency of thromboplastinogen in the blood It can, therefore, be designated as a congenital, hereditary hypothromboplastinogenemia It appears likely that the level of thromboplastinogen is relatively constant and that the severity of the condition depends on how low the level is fixed The variations of the bleeding tendency in the individual, however, are probably the result of other factors superimposed on the basic defect

Although the diagnosis of hemophilia is usually relatively easy, it may occasionally be extremely difficult The classical triad a congenital bleeding tendency in a male, positive family history, and a prolonged coagulation time of the blood is a useful guide but has limitations Not always is it possible to establish the complete triad

Frequently no hereditary history is obtainable and surprisingly often the coagulation time is equivocal Fortunately, the prothrombin consumption test promises to become an important means not only to aid in establishing a diagnosis, but also to estimate quantitatively the severity of the disease[34] In table 2 typical findings are recorded

TABLE 2 – THE PROTHROMBIN CONSUMPTION TEST

| | | *Prothrombin consumption time* in seconds* | | | |
|---|---|---|---|---|---|
| | | *Time after formation of a solid clot* | | | |
| | *Tube* | *15 min* | *30 min* | *45 min* | *60 min* |
| Normal | I | 6 | 24 | 23 | 25 |
| | II | | 7 | 23 | 28 |
| | III | | | | 14 |
| Thrombocytopenic purpura | I | 7 | 7 | 9½ | 10 |
| Platelets—30,000 | II | | 7½ | 9½ | 10 |
| | III | | | | 9 |
| Hemophilia (severe) | I | 7 | 8½ | 8½ | 8½ |
| Coagulation time—1 hr | II | | 8½ | 8 | 8½ |
| | III | | | | 8½ |
| Hemophilia (mild)** | I | 9 | 13 | 14 | 14 |
| Coagulation time—8 min | II | | 9 | 16 | 16 |
| | III | | | | 11 |

* The procedure of Quick[24] was used

** The patient had a positive family history and had a mild bleeding tendency since childhood

It should be remarked that this test has not had wide clinical trial and, therefore, its evaluation can only be tentative

The diagnosis of hemophilia requires clinical observation, a careful history, and laboratory study The bleeding of hemophilia is characteristically deep in contrast to the hemorrhage of purpura which is mostly limited to the skin and mucous membrane In hemophilia bleeding into muscles and joints is common Since this type of bleeding produces pressure, pain often is present while it is infrequent in purpuric hemorrhage Hemophilic bleeding may be indistinguishable from that caused by hypoprothrombinemia, but the two conditions are readily differentiated by the prothrombin time test

A hereditary history when obtainable makes the diagnosis certain The writer has seen a number of very mild cases in which the bleeding tendency is present but not severe enough to produce for instance, hemarthroses The clotting time, too, is either normal or only slightly prolonged If it were not for a positive family history which includes members who are unquestionably true hemophilics, the diagnosis could not be made with any degree of certainty Typical findings in 20 cases recently studied are summarized in table 3

TABLE 3.—SUMMARY OF FINDINGS IN 20 HEMOPHILIACS RECENTLY STUDIED

| Patient | Age (yr) | Coagulation Time (Lee White) (min) | Prothrombin Consumption (Quick) (sec) | First Hemorrhage | Hemarthrosis | Epistaxis | Transfusion | Hereditary History |
|---|---|---|---|---|---|---|---|---|
| 1 H J | 22 | 22 | 12½ | 6 m ıgotomy | + | Infrequent | + | + |
| 2 J K | 2 | 15 | 8 | Circ n | 0 | 0 | + | + |
| 3 D K | 2 | 25 | 12 | Circ n | + | 0 | + | − |
| 4 K F | 6 | 15 | 14 | 2 yr n of furuncle | 0 | One attack | 0 | + |
| 5 W G | 35 | 40 | 8 | 4½ y injury | + | Infrequent | + | + |
| 6 C W | 1½ | 25 | 10 | 1 yr ematoma | 0 | 0 | + | − |
| 7 R M | 3 | 10 | 13 | Circ n | + | 0 | + | + |
| 8 J B | 2 | 40 | 9 | Circ n | 0 | 0 | 0 | − |
| 9 J H | 8 | 15 | 12 | Ooz umbilicus | + | 0 | + | − |
| 10 T B | 3 | 11 | 9 | Circ n | 0 | 0 | 0 | − |
| 11 W C | 6 | 65 | 9 | 6 m ple bruises | + | 0 | + | − |
| 12 G S | 4 | 80 | 9½ | Circ n | 0 | 0 | + | − |
| 13 R A | 1 | 45 | 10 | Circ n | 0 | One attack | 0 | − |
| 14 R K | 39 | 22 | 10 | 7 m ing | + | Occasional | + | + |
| 15 T D | 8 | 8 | 11½ | 1 yr | 0 | 0 | + | + |
| 16 L H | 26 | 38 | 9½ | 1 yr ng from mouth | + | Frequent | + | − |
| 17 W S | 31 | 60 | 8 | 3 yr of mouth and teeth | + | Occasional | + | + |
| 18 N S | 33 | 30 | 9 | 3 yr foot | + | 0 | + | + |
| 19 L K | 13 | 90 | 8 | 6 m toma from blow | + | Occasional | + | + |
| 20 R B | 11½ | 22 | 12 | Circ... n | 0 | 0 | 0 | − |

* Quick, A J J Am Med Assn, 145 4, 1951 By permission of the American Medical Association

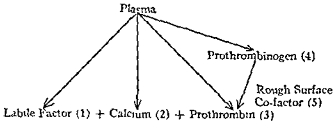
The laboratory offers invaluable assistance in the diagnosis of hemophilia. In all but a small number of hemophiliacs the clotting time is significantly prolonged provided the blood can be collected without admixture of tissue juice This may be difficult to achieve in young infants, and accounts perhaps for delayed diagnosis of this disease in children. The prothrombin consumption test promises to be much more informative and reliable The writer has applied the test to three newborn infants in whom the disease could be expected because of the family history. In one whose blood was studied on the fourth day of life, a prothrombin consumption typical of hemophilia was obtained Likewise in a second infant who bled following circumcision the prothrombin consumption test was low and has remained abnormal and the child subsequently showed signs of a hemorrhagic tendency. In the third boy the test has been consistently normal The prothrombin time and other hemostatic tests are normal in hemophilia It should be remembered, however, that the disease can be complicated by thrombocytopenia, hypoprothrombinemia, a circulating anticoagulant and other conditions Table 1 is helpful in differentiating hemophilia from other hemorrhagic conditions

**Acquired Hemophilic-Like Diseases.**—It is entirely reasonable to expect that the factor, thromboplastinogen, which in true hemophilia is reduced by an inborn defect, may be decreased in normal individuals analogous to acquired hypoprothrombinemia It appears likely that the case described by Joules and Macfarlane[31] of a woman with a hemophilic-like diathesis might well belong to this category In all such cases the platelet enzyme inhibitor (circulating anticoagulant) must be ruled out, before a diagnosis can be made

**Hypoprothrombinemia.**—According to the classical theory, prothrombin was considered to be a unitary substance, but the discovery in 1943 of a labile factor[25] which was found to be equally as necessary for prothrombin activity has led to a revolutionary revision of the concept of prothrombin Quick and Stefanini[41] found that the labile factor was not an accelerator of prothrombin conversion, but an actual component participating in the stoechiometric reaction to form thrombin They [42] further established that in addition to the free prothrombin, human plasma also contained a large amount of the prothrombin in a precursor state which for convenience can be designated prothrombinogen For the activation of the latter a rough surface and perhaps an additional factor tentatively designated cofactor are necessary The prothrombin complex can be presented diagrammatically, table 4

Prothrombin activity as measured by the one-stage method is dependent upon the concentrations of free prothrombin, the labile factor

TABLE 4 — THE PROTHROMBIN COMPLEX




| | *Labile Factor (1)* | *Calcium (2)* | *Prothrombin (3)* | *Prothrombinogen (4)* | *Co-factor (5)* |
|---|---|---|---|---|---|
| Effect of Storage | Decreased* | — | None | Decreased** | Increased? |
| Effect of heating to 60° C | Inactivated | | Inactivated | Inactivated | |
| Action of $Ca_3(PO_4)_2$ | Not adsorbed | — | Adsorbed | Adsorbed | Not adsorbed |
| Effect of Dicumarol | None | *** | Decreased | Decreased | None |
| Effect of vitamin K deficiency | None | *** | Decreased | Decreased | None |

* Rapidly in the absence of ionized calcium
** Becomes converted to active prothrombin (3)
*** Calcium requirement for optimum prothrombin time increased.

and calcium. The latter appears to be combined with a compound which is necessary for the reaction but the nature of this compound *and its rôle remains unknown It has been proven experimentally* that a prolonged prothrombin time can occur either from a decrease of free prothrombin or from the lack of the labile factor. Thus far no other factor has been found either experimentally or clinically which increases prothrombin activity in fresh plasma other than the concentration of free prothrombin itself

The rôle of prothrombinogen remains an enigma While it gradually becomes converted to the free state when oxalated or citrated plasma is stored in glass and is rapidly activated when blood is allowed to clot in glass, there is no evidence that any appreciable conversion occurs when coagulation takes place in a silicone-coated container Since coagulation in silicone-coated vessels perhaps approximates more closely the endothelial lining than does glass, it seems questionable whether the prothrombinogen plays any immediate rôle in physiological clotting It must further be remembered that only a part even of the free prothrombin is converted to thrombin during coagulation Apparently there exists a ratio of free to total prothrombin and as the total is reduced the free decreases proportionately Preliminary studies indicate that this occurs in Dicumarol therapy.

**The Congenital Types of Hypoprothrombinemia.**—Soon after the relationship between vitamin K deficiency and hypoprothrombinemia was established, Rhoads and Fitz-Hugh[19] described a case in which a hypoprothrombinemia was present from early life Neither the bleeding tendency nor the prothrombin level responded to vitamin K. They called the condition idiopathic although it probably was congenital. In 1943 Quick[11] discovered a new prothrombin factor which decreases rapidly in stored oxalated plasma, but remains unaffected by Dicumarol and vitamin K deficiency Quick stated that although a decrease of this factor (which he later designated as the labile factor) has been observed only in vitro, "it is entirely probable that clinical hypoprothrombinemia due to lack of this factor may occur" One year later Owren[21] found the first case resulting from a deficiency of the labile factor He named the condition parahemophilia, which unfortunately is a confusing term

In 1946 Quick[22] reported that he had found a fixed lowered prothrombin in a young healthy medical student For a period of several years the prothrombin time remained at 15½ to 16 seconds instead of 12 seconds which is the normal value He found that the subject's mother and sister also had this fixed prothrombin time of 16 seconds It showed clearly that the defect was both congenital and hereditary Later Quick[29] studied another family in which two boys with a bleed-

ing tendency had a prolonged prothrombin time. Significantly it remained consistently at 19 to 20 seconds. The defect seems certain to be congenital since the bleeding tendency was discovered in one of the boys when he was *circumcised at the age of 7 days. Vitamin* K had no effect on the prothrombin level of either family and in both the labile factor was normal. Surprisingly, when the blood of the first subject was added to an equal volume of the blood of one of the boys of the second family, the mixed blood had a normal prothrombin time.

From these results it became obvious that the hypoprothrombinemia of the medical student was not identical with that of the two boys of the second family. Further study disclosed that in the first case the total prothrombin was practically normal and that only the free was diminished In the second type both the free and the total were diminished The mutual correcting effect of the two bloods can be explained satisfactorily as follows: The blood of the first subject is *lacking in the co-factor necessary to convert prothrombinogen to* reactive prothrombin, whereas the second type blood contains the co-factor but is deficient in prothrombinogen On mixing the two bloods, the co-factor of type 2 converts enough of the prothrombino*gen of the first blood to bring the level of the reactive prothrombin in* the mixed blood to normal.

From the studies reported thus far, one can conclude that three types of conge[illegible] measured by the one-stage test [illegible] tor) is readily diagnosed, for [illegible] (deprothrombinized with tricalcium phosphate) to 9 volumes of the plasma to be tested, the prothrombin time becomes normal For establishing the diagnosis of the second type in which only the free prothrombin is reduced, *the following procedure is employed Blood is collected in* silicone-coated glassware and centrifuged at 4,000 R.P M in an Angle Centrifuge for 15 minutes to remove the platelets. The plasma is transferred to a glass test tube and allowed to clot *Under these conditions, the prothrombinogen is converted completely to free prothrombin* Since the removal of platelets stops the activation of [illegible] is changed to thrombin

[illegible]

of Quick. If the hypoprothrombin belongs to the second type, the total prothrombin will be the same as that of normal blood In the third type, the total is markedly diminished.

**Acquired Hypoprothrombinemia.**—*Preliminary studies indicate that vitamin K deficiency causes a decrease of both free and total*

prothrombin but does not affect the concentration of the labile factor. In Dicumarol poisoning both the free and total are diminished, but the mechanism whereby this is brought about remains undetermined. The labile factor is not affected by this drug [44] In experimental liver damage, Quick[38] obtained evidence that both prothrombin and the labile factor are reduced. This has been verified by Munro and his associates[29] who noted a decrease of both components after total hepatectomy Recently Sykes, Seegers and Ware[51] have also noted that Ac-globulin (which appears to be identical with the labile factor) is diminished after liver damage with chloroform.

Since the labile factor is rarely diminished clinically, the one-stage prothrombin test, which estimates free prothrombin, has in the decade and a half since its introduction into the clinical laboratory yielded consistently reliable results for diagnosis, prognosis and control of therapy Nevertheless for accurate scientific studies, new methods, particularly the procedure of Quick and Stefanini[35] in which the prothrombin is adsorbed by tricalcium phosphate and eluted with sodium citrate, are recommended to complement but not to supplant the one-stage method

**Hypofibrinogenemia.**—The congenital type of hypoprothrombinemia is not only an exceedingly interesting clinical entity, but it has served as a source of valuable information. Pinniger and Prunty[33] found that the platelets agglutinated when the blood was placed in a glass test tube, and that thrombin was formed Recently Quick[40] noted that the prothrombin consumption was very rapid in afibrinogenemic plasma which is explained by the absence of fibrin which adsorbs thrombin and slows the thrombinogenic cycle Of greatest importance is the observation that patients with complete absence of fibrinogen often have a relatively mild hemorrhagic diathesis The patient of Pinniger and Prunty is menstruating regularly Although the flow is heavy, it stops spontaneously at the end of about 7 days At times these patients will have little bleeding following such trauma as results from tooth extraction, which in a hemophiliac invariably causes serious hemorrhage

The diagnosis is readily made The blood is collected and oxalated as in the prothrombin time test If no clot forms on recalcification and on the addition of thromboplastin the absence of fibrinogen is suggested The plasma is then mixed with an equal volume of rabbit plasma deprothrombinized with tricalcium phosphate and the prothrombin time of the mixture determined If the condition is uncom-

ing hypoprothrombinemia is present In determining the fibrinogen, it is to be recommended that it be done by two methods In the first procedure the fibrinogen should be clotted by the addition of thrombin and in the second the fibrinogen should be precipitated with half saturated sodium chloride A simple means to test whether any fibrinogen is present in blood, is to heat the oxalated plasma at 60° C for 10 minutes At that temperature, the albumen and globulin are not coagulated

**Platelet Deficiency in Vasoconstrictor Principle.**—Since platelets supply a vasoconstrictor substance on lysis, it is theoretically possible that under certain pathological conditions platelets may be produced which lack this pressor agent The writer has seen a patient who for several years has had a platelet count consistently over two million Paradoxically, he has a hemorrhagic tendency On one occasion he has required several transfusions before the bleeding following the extraction of a tooth was stopped He develops spontaneous deep hematomas His coagulation time, bleeding time, prothrombin concentration and prothrombin consumption and fibrinogen are normal There is a definite likelihood that his platelets are functionally deficient The bleeding and thrombocytosis began shortly after splenectomy As soon as a reliable clinical method for determining the vasoconstricting agent in platelets is developed, the functional properties of this patient's platelets will be studied

Since the vasoconstrictor agent in platelets is an established fact, it is desirable that a suitable name for this substance be selected Reid[47] has proposed the name "thrombocytin" while Rapport and his associates[46] have designated the pressor factor in serum, which is probably identical with the platelet agent, "serotonin" Perhaps a compromise name, "thrombotonin" might be selected This name suggests both the source and action of the compound

**Heparinemia.**—Although heparinemia can readily be produced in dogs either by anaphylactic shock or by injection of peptone, the appearance of heparin in human blood is uncommon It is difficult to find in the medical literature any evidence that heparin has ever been found to be the primary cause of any hemorrhagic disease in man Jorpes,[20] to be sure, cites one condition, urticaria pigmentosa, in which mast cells accumulate about the capillaries of the skin Since these cells are rich in heparin, and since petechial hemorrhages are common in this disease, he implies that a causal relation may exist

Recently Allen and Jacobson[3] found that in certain purpuric conditions such as resulted from exposure to ionizing radiation the appearance of a heparinoid substance in the blood can be detected This

substance is determined by the protamine titration developed by Allen and his co-workers[4]

To establish the presence of heparin in blood, the titration of plasma with varying concentrations of thrombin according to the method developed by Quick[16] is simple and reliable. With this test the amount of heparin can be estimated with a high degree of accuracy

**Recapitulation.**—The hemorrhagic diseases can be grouped into two general classes The first consists of those entities in which the defect is in the vessel itself while in the second the fault is traceable to the blood The second class is clinically the more important since it includes the more common types of the hemorrhagic diseases These may be subdivided into the hypothromboplastinemias, hypoprothrombinemias, and hypofibrinogenemias. The prothrombin consumption test determines the existence of the first, while the prothrombin time procedure establishes the presence of the second

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## Chapter 4

# TREATMENT OF THE HEMORRHAGIC DISEASES AND SURGICAL HANDLING

WHENEVER hemorrhage is encountered, the decision has to be made *whether it is purely traumatic in origin or whether it is brought on* or accentuated by a basic hemostatic defect. Generally, no difficulty is experienced in recognizing bleeding conditions arising solely from trauma, and both the internist and the surgeon have learned from long experience the various means for controlling this type of hemorrhage Treatment runs the wide gamut from an injection of morphine to control hemoptysis to ligation of a severed or corroded artery Obviously the subject of hemorrhage in the normal patient is too extensive for adequate presentation, and discussion will, therefore, be limited to the control of bleeding in the hemorrhagic diseases

The first prerequisite for the rational treatment of hemorrhage is an accurate diagnosis In the preceding chapter an outline of the known hemorrhagic diseases was presented and their characteristics discussed in order to provide a guide for diagnosis and treatment. The clinical approach to the bleeding condition is the same as to any other diseases A careful history is essential and in obtaining it, a specific inquiry should be made in regard to the presence of a bleeding tendency in the individual and in his family. Attention to minor signs and symptoms is important Repeated nose bleeds may sometimes serve as the clue to the diagnosis of pseudohemophilia and telangiectasia Easy bruising in an infant may be noted by parents for a long time before a diagnosis of hemophilia is made

Often serious difficulties are encountered when an attempt is made to decide whether hemorrhage from a known lesion is accentuated by a concomitant coagulation defect A specific instance is the bleeding of esophageal varices in hepatic cirrhosis with an accompanying mild hypoprothrombinemia A second example is hemorrhage in pulmonary tuberculosis and a co existing state of hypoprothrombinemia Such complications may lead to serious consequences. Thus, the development of a gastric ulcer in a patient with pseudohemophilia may give rise to bleeding that will require heroic efforts to prevent the patient from becoming exsanguinated

In the diagnosis of the hemorrhagic diseases, the laboratory is

indispensable, but oddly enough only three tests are needed in the majority of instances to establish a diagnosis. The three tests are: (1) the bleeding time, (2) the prothrombin time and (3) the prothrombin consumption determination. The coagulation time yields little if any additional information, but it has occupied an honored position in the list of laboratory procedures so long that few will have the temerity to omit it. When carefully performed, the Lee-White procedure serves as a screening test. A prolonged coagulation is always significant, but a normal clotting time does not exclude serious coagulation defects in the blood. It cannot be emphasized too vigorously that normal bleeding and clotting times offer no assurance that the patient has a normal hemostatic mechanism and that he will not bleed postoperatively. A careful evaluation of the patient's answer to the simple question: "Do you bleed easily?" may be far more significant than a carefully performed coagulation time test.

**Hereditary Telangiectasia.**—Since no means have been found to prevent or cure this disease, therapy is mainly directed to the control of bleeding by local means. Nose bleed can often be stopped by the insertion of a small balloon which on inflation acts as a pressure tampon.[20] Various agents have been recommended for destroying the nevi. These have been reviewed recently by Figi and Watkins.[11] These authors conclude that electro-coagulation of the lesion is the most effective means to stop nasal bleeding.

A hopeful note in therapy is found in the report of Kushlan[22] that rutin, a flavone glucoside, markedly improved the bleeding tendency in telangiectasia. Cope and Grover[8] have also obtained favorable results in one case. Should these findings be substantiated by studies on a larger series of cases, it will mark the first encouraging advance in the treatment of this troublesome disease. It now appears that the early reports were probably too optimistic. Glass[16a] recently reported that three cases of telangiectasia failed to respond to this therapeutic agent.

Since the lesions are superficial and the coagulation of the blood[17] entirely normal, no trouble is encountered when a patient with this disease undergoes surgery. A transfusion of blood will have no effect on the basic hemostatic defect of the disease, but may be beneficial since these patients frequently have a secondary microcytic anemia. Adequate administration of iron is also essential in treating the anemia.

**Pseudohemophilia.**—The treatment of hemorrhage in this disease is most unsatisfactory especially if the bleeding comes from an inaccessible location such as the stomach. Transfusions of blood or plasma are apparently entirely ineffective except for maintaining the

5

hemoglobin above the safety level and counteracting shock Splenectomy has given disappointing results, and may be a hazardous procedure [27] The most common forms of hemorrhage in this disease are epistaxis and bleeding after extraction of teeth Both are amenable to local measures such as pressure tampons. The use of fibrin foam, sponge gelatin or oxidized or absorbable cellulose with or without thrombin should have therapeutic value, but no extensive studies apparently have been made. McLaughlin[28] used *gelfoam* (sponge gel) on one patient with partial success

It is interesting that the bleeding time which is the best criterion to judge the severity of the hemorrhagic tendency does not remain constant but shows marked variations The cause for these variations is not known If means can be found to shorten the bleeding time, a marked advance in the treatment of pseudohemophilia will undoubtedly result

A serious problem arises when a subject with this disease requires surgery since the severity of postoperative bleeding is unpredictable and the control unsatisfactory If an emergency operation is required, it should be recognized that many transfusions may have to be given before bleeding is permanently stopped

**Purpura.**—Capillary hyperpermeability which gives rise to superficial skin and mucous membrane hemorrhage may occur without any demonstrable qualitative or quantitative change in the platelets, or it may be accompanied by a marked thrombopenia in which case the hemorrhagic tendency is usually much more severe

In the non-thrombocytopenic purpuras, the control of hemorrhage is usually not a serious problem In the Schonlein-Henoch Syndrome gastro-intestinal bleeding may be troublesome, but it is generally overshadowed by other symptoms and signs which are more sinister Often the abdominal colic, the rigidity and the distension are so extreme, that it becomes difficult to decide if the patient should be transferred from medical to surgical care This is particularly true in the case of infants in whom intussusception is a recognized complication of Henoch's disease Only recently Benson and Martmer[5] have reported such a case and have given references to previous ac-

non-

thrombocytopenic type If the bleeding is severe enough to cause a marked anemia, transfusion of whole blood is indicated If surgery is necessary, it can be carried out without trepidation, insofar as the danger from hemorrhage is concerned It should be mentioned that a hereditary condition of capillary weakness is apparently not uncommon Davis[11] calls this state a hereditary familial purpura sim-

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There is no specific treatment for purpuric bleeding of the non-thrombocytopenic type If the bleeding is severe enough to cause a marked anemia, transfusion of whole blood is indicated If surgery is necessary, it can be carried out without trepidation, insofar as the danger from hemorrhage is concerned It should be mentioned that a hereditary condition of capillary weakness is apparently not uncommon Davis[11] calls this state a hereditary familial purpura sim-

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The common causes of thrombocytopenia with normal or hyperplastic bone marrow are:

1) Infection (Virus, bacteria and occasional higher forms such as plasmodia, *etc.*).
2) Blood dyscrasias (Particularly acute leukemias).
3) Allergy (Drugs, foods, synthetic hormones).
4) Diseases of the liver
5) Ionizing radiation

This list serves as a guide for determining the agent and factor responsible for the decrease in the platelet count and the hyperpermeability of the peripheral capillaries Sometimes it becomes difficult to decide whether one agent is responsible for both the effect on the platelets and on the capillaries It is not uncommon to observe a child with an acute lymphatic leukemia to have a negative tourniquet test, a normal bleeding time and no purpura in spite of a platelet count below 25,000 As soon, however, as aminoptyrin (a folic acid antagonist) is administered a marked bleeding tendency may develop, petechia will appear and both the tourniquet and the bleeding time test become positive without any change in the platelet count Observations such as these lead one to conclude that although thrombocytopenia and purpuric bleeding are closely associated, they are separable factors

The first step in the treatment of thrombocytopenic purpura is to remove the cause when possible This is readily accomplished when the condition is caused by a drug or food to which the patient has developed sensitivity The clearing of an infection may cure the purpura, but there may be a considerable lag in the time required for the purpura to disappear and the bleeding to stop

Although transfusions are frequently given, there is no good evidence that they have any beneficial effect other than combating anemia Lawrence, Valentine and Adams[24] found that the platelet count could not be elevated in thrombocytopenia by even large volumes of fresh blood It is a common experience that the bleeding tendency likewise is little altered by the administration of fresh blood It appears logical, therefore, to employ transfusions primarily to correct the anemia, and to be guided solely by the erythrocyte count and the hemoglobin level as to when they should be given Many types of treatment have been suggested from time to time The older of these measures are reviewed by Quick[37] They include splenic irradiation, snake venom, vitamins C and P, parathyroid hormone, and drugs related chemically to epinephrine In spite of the initial enthusiasm with which these agents were first reported, subsequent critical study apparently has found them wanting Of probable great

significance is the recent report of Meyer, Miller and Bethell[2*] that three patients with idiopathic thrombocytopenic purpura had a prolonged clinical and hematological remission after a single course of therapy with ACTH. A dose of 100 mg. was given for 8 to 13 days. Similar results have also been published by Robson and Duthie[**].

Recently Allen and his associates[3] have reported promising results with toluidine blue and protamine sulfate. Both these substances antagonize or neutralize the action of heparin. While there is no proof that actual heparin is present in the blood, Allen has by means of his test, the protamine titration, adduced evidence of a condition which he has named a heparinoid state. In many instances, he has found that a bleeding condition in which the protamine titration is elevated would respond to these two drugs given intravenously. Allen and his co-workers[4] recommend that a daily dose of 7 to 10 mg. of toluidine blue per kilogram of body weight be given intravenously for 3 to 6 days. The dose of protamine sulfate is 150 mg. Both must be given in several hundred cc. of physiological saline solution and at a very slow rate. Allen and his group have found that toluidine blue is ineffective in the idiopathic type of thrombocytopenic purpura, and this also has been the observation of other workers.[14] From these limited studies, it appears that in certain hemorrhagic conditions these drugs effect a dramatic curative action, while in others including cases with thrombocytopenia no benefit is obtained.

Vitamin P and related flavone compounds, particularly rutin, have been tried therapeutically for controlling purpuric bleeding with equivocal results. Recently a more favorable response has been reported in a group of conditions named radiation disease. These studies are reviewed by Levitan.[26]

The writer has observed in a small number of cases of thrombocytopenic purpura in children a favorable response to folic acid. Curiously, the bleeding stopped long before the platelet count began to increase. The series is still too small, however, to warrant drawing any definite conclusions.

**Hemophilia.**—Until very recently, attempts at therapy in hemophilia could be best described by the simple Anglo-Saxon word—"groping." All treatment had to be on an empirical basis since the nature of the disease was not understood. Nevertheless, a number of observations were made which have been of immeasurable value. The most important of these was the finding that transfusions definitely assisted in controlling the bleeding. It is interesting to note that Lane[23] over one hundred years ago gave a blood transfusion to a hemophiliac, who had been operated on for correction of squint, and he

thereby controlled the serious postoperative bleeding In 1922 Stegemann[19] summarized the reports of 12 European workers who obtained good results using transfusions in hemophiliacs undergoing surgery Nevertheless, the failures, which rarely are reported, unquestionably far outnumber the relatively few reported successes which have been summarized in the papers of Mertz and Meiks,[29] Schiller and his co-workers[48] and Craddock and his associates.[9] The latter makes the significant statement: "Emphasis is placed on the failure of the *coagulation time to indicate the severity of the hemorrhagic tendency* or the degree of response to treatment "

A rational approach to a reliable control of hemophilic bleeding is contingent on understanding the nature of the disease. It has been recognized by many investigators that hemophilic blood lacks a factor necessary for the conversion of prothrombin to thrombin. Some like Bendien and Creveld,[4] Patek and Taylor and their associates,[32 33 34] Lenggenhager[25] and others believed that this factor is a plasma constituent, whereas many other workers including Howell,[19] Fonio[15] and Brinkhous[6 7] maintained that the prothrombin activator or thromboplastin is supplied by the disintegrating platelets and that in hemophilia the platelets are broken down too slowly to meet the hemostatic demands Quick who had also shared these views prior to 1947, showed that the hemophiliac has a congenital lack of the thromboplastin precursor found in the plasma, which he named thromboplastinogen [41] His experimental findings showed that this precursor is activated by a platelet factor In other words, the platelets do not supply thromboplastin but activated it from a plasma precursor By means of the prothrombin consumption test, he was able to obtain a fairly quantitative estimation of the thromboplastinogen concentration

On the basis of Quick's studies, one can postulate that the basic defect in hemophilia is a deficiency of thromboplastinogen which accounts for a low production of thrombin This not only causes a delayed clotting time but a very incomplete lysis of platelets, thereby causing an inadequate liberation of the vasoconstrictor on which effective hemostasis depends *The abnormal bleeding is not due to* a failure of a clot to form, but to a lack of the clot to supply the pressor agent By transfusion of normal blood, thromboplastinogen is supplied and the basic defect partially corrected

From personal observations of various kinds of bleeding encountered in hemophilia, the writer has come to the conclusion that at least 90 per cent comes from rupture of minute arteries and arterioles The larger arteries having thicker and stronger walls as well as the protection of their deeper location are rarely involved except in major

accidents. The hemophiliac has no trouble controlling capillary hemorrhage as evidenced by the fact that the bleeding time, which measures the capillary response, is normal Significantly, the bleeding time tends to remain normal until the cut is about 6 mm. deep,[47] when presumably arterioles are severed.

The hemophiliac has two normal hemostatic responses the initial or primary vascular contraction immediately after injury and the gluing effect when endothelium is pressed together, which is particularly effective in controlling capillary bleeding. The first is often not sufficient in itself Thus, bleeding resulting from the extraction of a tooth is often temporarily controlled, but after the initial contraction has passed, bleeding resumes because the thrombin phase is defective Undoubtedly, the adhesiveness of compressed endothelium is the most important means whereby most of the bleeding in hemophilia is eventually stopped and this fact helps to rationalize local therapy

The first step in controlling hemophilic hemorrhage locally is the application of cold and pressure By means of cold, preferably an ice bag, the vessels in the area are contracted and the blood pressure decreased Under these conditions there is a greater opportunity for the injured vessel to be sealed by endothelial adhesiveness Under no conditions should heat be applied It is not uncommon to see a minor bleeding become massive as the result of using heat locally The application of pressure is more limited in scope, since it is ineffective unless the location of the hemorrhage is suitable A pressure bandage promptly applied to a hemarthrosis, or to a beginning hemorrhage into a muscle of an arm or leg often effectively hastens stanching and limits the size of the hemorrhage Simple prolonged pressure upward against the side of the nose to bring the turbinates against the septum occasionally is enough to stop epistaxis The cardinal points of local treatment are cold, pressure and rest If these measures fail to bring the bleeding under control, a transfusion of fresh or lyophilized plasma should be given The effect of this measure should be determined by the prothrombin consumption test as will be discussed later Since evidence is accumulating which makes it appear probable that transfusions may sometimes cause the appearance of a circulating anticoagulant, (an antagonist to the platelet enzyme) it seems judicious to give blood or plasma only when there is definite need for it

One of the most serious sequelæ of interstitial hemorrhage is pressure atrophy If there are clinical signs that this is occurring, the blood must be aspirated after the patient has been prepared with a plasma transfusion Subperiosteal hemorrhage may cause bone atrophy and pseudotumors which at times may be mistaken for bone sarcoma since they simulate the roentgen changes of the later accord-

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TABLE 5.—THE PROTHROMBIN CONSUMPTION TIME TEST AS A MEANS TO TEST THE EFFICACY OF NORMAL BLOOD TO CORRECT THE DEFECT OF HEMOPHILIC BLOOD IN VITRO AND IN VIVO

| | Clotting Time | Tube | Prothrombin Consumption Time in Seconds* — Time after formation of a solid clot: 15 min | 30 min | 45 min | 60 min |
|---|---|---|---|---|---|---|
| Normal (A J ) | 6 min. | I | 6 | 24 | 28 | 30 |
| | | II | | 10 | 25 | 27 |
| | | III | | | | 15 |
| Hemophiliac (L K ) | 65 min | I | 8 | 8½ | 8½ | 8½ |
| | | II | | 8 | 8 | 8½ |
| | | III | | | | 8 |
| Hemophiliac (L K ) 9 vol.** | 12 min | I | 13 | 14 | 14½ | 14½ |
| | | II | | 12½ | 13 | 13 |
| Normal (A J ) 1 vol | | III | | | | 12 |
| Hemophiliac (L K ) After transfusion of 250 cc of lyophilized plasma | 6 min | I | 6 | 10 | 10 | 11 |
| | | II | | 7 | 13 | 13 |
| | | III | | | | 9½ |

* The procedure of Quick was used

** The blood was mixed in a test tube

It will be observed that the patient's blood responded to normal blood and plasma both in vitro and in vivo While the transfusion did not bring the prothrombin consumption time to the normal level, it did apparently elevate it sufficiently to meet the hemostatic demands After a week at this level, the transfusions were stopped No more oozing occurred than is usually observed in a normal subject Similar results have been obtained in several other orthopedic operations and in one appendectomy in which a normal appendix, but a retroperitoneal hemorrhage, was found

Obviously the series is too small to put an unqualified stamp of approval on the prothrombin consumption test It is to be emphasized that the test will require much study before its reliability is fully established It seems certain that it does not measure solely the thromboplastinogen concentration It appears to be influenced by a factor which inhibits the platelet enzyme Thus, one should consider that the test in its present form measures the composite thromboplastic activity Interestingly enough this activity varies in normal individuals, and therefore some donors are more satisfactory for correcting hemophilic blood than others This is illustrated in table 6

On examining the results of table 6, it is clearly seen that Subject M S 's blood elevated the prothrombin consumption time of the hemophilic blood definitely more than did the blood of A J in spite

ing to Echternacht.[13] For a summary of bone complications, the article of this author as well as the paper of Ghormley and Clegg[14] should be consulted.

The neurological complications are even more serious. Bleeding into the brain or spinal cord is usually difficult to cope with; but occasionally an intrepid decision to operate to relieve the pressure is rewarded by a brilliant result, as for instance one recorded by Schiller and his associates[48] who obtained a partial recovery of a paraplegia, due to a subdural spinal hematoma, by an extensive laminectomy. The various neurological complications of hemophilia with a review of the literature are presented by Aggeler and Lucia.[1]

Not only is it essential to stop hemorrhage, but it is equally as important to avoid permanent damages such as ankylosis and muscle atrophy Immediately after the bleeding into a muscle or joint has subsided, heat should be cautiously applied to hasten the readsorption of extravasated blood. Later, diathermy, passive exercise, and light massage yield valuable results When recovery is complete, active exercise is recommended to maintain good circulation, for in that manner the joint is brought into its normal state Swimming is particularly to be recommended Physiotherapy is unfortunately still inadequately employed in the treatment of the sequelæ of hemophilic bleeding Prolonged splinting and rest for hemarthrosis explains many of the ankyloses that one sees so frequently

One observation that the writer has failed to find in any discussion of hemophilia is that when bleeding has occurred into a joint, especially if repeatedly, attacks of arthritis, generally caused by strain or overwork of the joint, are common There is even evidence at times of effusion into the joint It is difficult to differentiate between this inflammatory state and true hemarthrosis Blood transfusions are of no value in the treatment of the arthritis

Surgery in hemophilic subjects presents an interesting and important problem While a surprisingly large number of hemophiliacs have survived operations, it is no exaggeration to say that multiple transfusions and a kind fate tipped the balance in their favor In none of these cases was any control used other than the coagulation time, which Craddock and his associates[9] justifiably condemn as indicating neither the severity of the condition nor the degree of response to treatment With the development of the prothrombin consumption test, a new means for measuring the response to therapy has become available While its limitations should not be underestimated, one cannot deny that it represents an important step forward. In table 5 the application of this test to the practical problem of preparing a hemophilic for an extensive bone operation is presented

given. It appears that the hemostatic potency of blood is increased rather than decreased by anemia. It should also be remembered that most hemophiliacs have a remarkable ability to regenerate erythrocytes.

Infection complicates the problem of hemostasis in hemophilia. The extraction of an infected tooth for instance in a hemophiliac is always serious. Fortunately with the availability of the antibiotics, the control of infection is now usually possible Granulation tissue may frequently be the cause of considerable bleeding and may have to be cauterized and destroyed to stop the oozing of blood

It should be emphasized that even though many operations have been successfully carried out on the hemophiliac, one must never lose sight of the hazard Even though it is fairly certain that the basic defect of the disease is lack of thromboplastinogen, a substance which normal blood contains in excess of its own minimal requirement for hemostasis, the administration of normal blood to correct the deficiency is sometimes not enough Before thromboplastinogen becomes available it must be activated by a platelet factor and the latter is counteracted by an inhibitor which is a variable even in normal blood The control of this variable is not understood Because of these uncertainties it seems best to limit surgical procedures to emergencies, and not to undertake elective operations unless reliable laboratory facilities are available

**Hypoprothrombinemia.**—The therapeutic approach to the bleeding caused by diminished prothrombin is simple. The objective is to bring the level above 30 per cent as measured by the one-stage method It has been demonstrated both experimentally[42] and clinically[43] that a prothrombin time of 20 seconds (using dehydrated rabbit brain as thromboplastin) is the critical value at which the hemorrhagic tendency manifests itself The prothrombin level can be elevated by giving plasma intravenously, or by vitamin K provided the hypoprothrombinemia is caused by a deficiency of this vitamin

The response to vitamin K in uncomplicated avitaminosis K is truly remarkable Collentine and Quick produced a drastic hypoprothrombinemia in a dog by means of an intraabdominal biliary fistula produced by a cholecystnephrostomy In the course of two months the prothrombin time dropped from a normal of 6 to 75 seconds On giving vitamin $K_1$ the prothrombin was promptly restored, but after the supply of the vitamin became exhausted the prothrombin rapidly fell to the previous low level In table 7, the effect of natural vitamin K and of menadione are presented.

The results clearly indicate that natural vitamin $K_1$ is much more effective than the synthetic product, menadione Miller *et al*,[30] in

TABLE 6 —VARIATIONS IN NORMAL PLASMA IN ITS EFFICACY TO CORRECT THE HEMOPHILIC DEFECT

| | Tube | *Prothrombin Consumption Time in Seconds — Time after the formation of a solid clot* 15 min | 30 min. | 45 min | 60 min |
|---|---|---|---|---|---|
| Normal (A J.) | I | 6 | 24 | 23 | 21 |
| | II | | 11 | 28 | 30 |
| | III | | | | 13 |
| Normal (M S) | I | 7 | 15 | 14½ | 13½ |
| | II | | 9 | 20 | 21 |
| | III | | | | 12 |
| Hemophiliac (W S) | I | 7 | 8 | 8 | 8 |
| | II | | 8 | 8 | 8 |
| | III | | | | 8 |
| Hemophiliac (W S) 19 Vol | I | 9 | 10½ | 10½ | 11 |
| | II | | 10 | 10 | 10 |
| Normal (A J) 1 vol | III | | | | 10 |
| Hemophiliac (W S) 19 vol | I | 11 | 12½ | 12½ | 12½ |
| | II | | 13 | 13 | 12½ |
| Normal (M S) 1 vol | III | | | | 11 |

of the fact that A J 's prothrombin consumption time was higher than that of M S Without attempting to explain these seemingly paradoxical results, the practical conclusion can be drawn that the antihemophilic potency of a normal plasma can only be assayed on hemophilic blood It should be remarked that none of the antihemophilic globulin fractions have been assayed by this method. The ability of an agent to bring a prolonged coagulation time to normal is no measure of its true antihemophilic potency

Transfusion is most effective if given immediately before or even during the operation The object is to increase the thromboplastinogen content to as high a level as is feasible in order to achieve permanent hemostasis immediately Small amounts of blood or plasma or plasma fractions may be enough to bring the coagulation time to normal but not enough to influence the prothrombin consumption The coagulation time, therefore, is most misleading and often creates a false sense of security

Plasma is far more valuable in combating hemophilic hemorrhage than is whole blood Since the thromboplastinogen is contained only in the plasma, one volume of the latter is equivalent to two volumes of whole blood Another great advantage is that much larger volumes of plasma can be given when the need arises than of whole blood Only when the anemia becomes fairly severe, should whole blood be

administering vitamin K to the mother prepartum, the incidence of hypoprothrombinemia and bleeding as a result of it is now very low It is more common at present to encounter hypoprothrombinemia caused by drugs such as certain sulfonamides and antibiotics which depress the bacterial flora of the intestines and thereby curtail the available vitamin K

When an operation has to be done on a very young infant, vitamin K (preferably the natural $K_1$) should be given pre-operatively and repeated daily after the operation until the baby again feeds regularly.

**Cholemic Bleeding.**—Postoperative hemorrhage in the jaundiced patient prior to 1936 was not only an enigma, but an exceedingly serious problem When vitamin K even in crude form was made available, this type of bleeding became only of historic importance Synthetic vitamin K preparations for both oral and parenteral administration are available commercially. They should be given before the operation, and their effectiveness determined by the prothrombin time If the prothrombin level fails to be raised to the 40 per cent level, a plasma transfusion should be given before the operation A poor response to vitamin K usually indicates liver damage

this is due directly to the low level of prothrombin It is much more likely to be a purpura, especially of the thrombocytopenic type, superimposed on the basic prothrombin deficiency As already mentioned Whitesell and Snell[91] have recently reported that thrombocytopenia is not uncommon in liver disease One can be reasonably certain that whenever the bleeding time becomes prolonged and typical superficial skin hemorrhage occurs in hypoprothrombinemia or hemophilia, the primary condition is complicated by an additional factor.

The hypoprothrombinemia resulting from liver disease is usually not severe enough to cause a frank hemorrhage tendency Probably the one exception is acute yellow atrophy Vitamin K will not elevate the prothrombin level Therefore, if surgery is required, the prothrombin concentration must be elevated and maintained by means of plasma transfusions

**The Hypoprothrombinemia Caused by Dicumarol.**—With the extensive use of this drug in therapy, it is not surprising that the number of severe hemorrhages and even fatal hemorrhages caused by it are increasing Duff and Shull[12] have collected a record of 23 deaths from bleeding caused by this drug With better control of dosage based on daily prothrombin time determinations, the incidence of abnormal bleeding would be greatly reduced Likewise by following

TABLE 7 —THE COMPARATIVE EFFECT OF NATURAL VITAMIN $K_1$ AND MENADIONE IN RESTORING THE PROTHROMBIN LEVEL IN A DOG WITH VITAMIN K DEFICIENCY

| *Time after administration hr* | *Prothrombin time in seconds* | | | | |
|---|---|---|---|---|---|
| | *Menadione* | | | *Vitamin $K_1$* | |
| | *1000 gammas* | *100 gammas* | *50 gammas* | *125 gammas* | *100 gammas* |
| 0 | 75 (½)* | 80 (½) | 55 (½) | 70 (½) | 40 (1) |
| 1 | 75 (½) | — | — | — | 42 (1) |
| 2 | 30 (2) | 60 (½) | — | — | 18½ (3½) |
| 3 | 10 (20) | 37 (1) | — | — | 8 (40) |
| 4 | 8 (40) | 25 (2) | 22 (2½) | 6 (100) | 7 (50) |
| 5 | 8 (40) | 19 (3) | — | — | — |
| 24 | 8 (40) | 10 (20) | 10½ (17) | 6 (100) | 6 (100) |
| 48 | 8 (40) | 12½ (9) | 16 (4½) | 7½ (45) | 8 (40) |

* The figures in parenthesis are the concentrations of prothrombin in per cent of normal

The experiments were carried out on a dog weighing 14 kg Several months earlier a cholecystnephrostomy had been performed

confirmation of older studies have also found that the natural product is much more potent in antagonizing the hypoprothrombinemia of Dicumarol than the synthetic vitamin K compounds. These differferences are not as apparent when chicks are employed as the test animals [44]

Quick and Stefanini[43] have recently developed a procedure whereby the prothrombin of plasma can be concentrated by means of adsorption with tricalcium phosphate and elution with sodium citrate With this method, these investigators have been able to prepare a solution of prothrombin of such strength that 10 cc had the potency of 200 cc of plasma as tested on its ability to elevate the prothrombin in Dicumarolized dogs This concentrate has not yet been employed in humans, but there appears no contraindication to its use

**Congenital Hypoprothrombinemia.**—The three types of congenital deficiency of prothrombin discussed in the preceding chapter, although differing basically require the same treatment None of the three show any response to vitamin $K_1$ but in all the hypoprothrombinemia is temporarily but promptly corrected by plasma transfusion In the type Owren[31] described, the plasma must be fresh since the lacking agent is the labile factor which diminishes in plasma when stored When the hypoprothrombinemia is very severe the patient may require frequent plasma transfusions Thus, the patient reported by Hagen and Watson[17] who has a prothrombin time of about 50 seconds requires a weekly transfusion, whereas the two boys studied by Quick[38] whose prothrombin is 19 seconds only rarely have hemorrhages and have required only a few transfusions

**Hemorrhagic Disease of the Newborn.**—With the improvement of the diet of the general population and the rather wide practice of

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Every patient that is put on Dicumarol therapy should be instructed to watch his urine for any sign of blood. Hematuria appears to be the most common and earliest form of bleeding. At the first indication of bleeding the administration of the drug should be stopped. If the hypoprothrombinemia is marked, the patient should be given a relatively large dose of vitamin $K_1$ oxide[30] or a water-soluble synthetic product such as Hykinone (2-methyl,-1,4-naphthohydroquinone, 3-sodium sulfonate). The usual amount given intravenously is 50 to 60 mg. For a comprehensive treatment of this subject the articles of Quick[38,40] and of Thompson and Hilferty[50] should be consulted

The use of Dicumarol prepartum is exceedingly dangerous. Quick[39] found that in dogs, the fetus was far more affected than the mother and that death of the puppies occurred shortly after birth as a result of massive internal hemorrhage with dosages that only moderately lowered the prothrombin of the maternal blood. Similar findings have been observed by Kraus and his associates[21] in the human, and Sacks and Labate[46] have reported the death of a human fetus from hemorrhage caused by Dicumarol It is clear, therefore, that Dicumarol should never be given before the baby is born. The drug is safe when given postpartum since the amount excreted in the milk is too small to affect the baby

**Recapitulation.**—The treatment of hemorrhage, in states with defective hemostasis, is local in some diseases such as hereditary telangiectasia, and systemic especially in those in which the cause is a deficiency of a clotting factor which can be corrected In hemophilia the lack of thromboplastinogen can temporarily be rectified by the transfusion of blood or plasma, thereby establishing a condition sufficiently favorable to permit the performance of a major operation The correction of the defect is measurable by the prothrombin consumption test In hypoprothrombinemia, vitamin K will correct the defect if it has been caused by a deficiency of this vitamin In congenital hypoprothrombinemia, and when caused by liver damage, vitamin K is ineffective, and only transfusion of plasma will elevate the prothrombin above the hemorrhagic level In Dicumarol poisoning, vitamin K can increase the prothrombin concentration, but its effect is relatively weak and large doses are required The critical level of prothrombin at which a hemorrhagic tendency appears in man is approximately 20 per cent of normal For surgical safety, a level of 40 per cent or higher is required.

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clots in the venous circulation, and I regard the occurrence of the first (in the pulmonary artery) as a sure sign that somewhere in the venous system, an older blood clot is to be found" (Author's translation.)

Another milestone was reached when Hayem[10] and Bizzozero discovered that platelet agglutination and disintegration initiated fibrin formation Eberth and Schimmelbusch[8] made the additional observation that when the circulation was slowed, platelets left the center of the stream, migrated to the periphery and became responsible for the formation of a thrombus Forty years later Aschoff[1] reemphasized the importance of the slowing of the circulation and the agglutination of platelets as causal events in thrombosis He enumerated the variables which have been widely accepted as the important factors in thrombosis They are: (1) changes in the coagulability of the blood, (2) changes in the blood elements, particularly in the power to agglutinate, (3) changes in the blood flow both in regard to speed and the formation of eddies, and (4) changes in the vessel wall In this country, Homans,[14] a pioneer worker in this field, came to similar conclusions Mention should also be made of the important contributions of Welch to the histopathology of thrombosis and his masterly presentation of the subject[22] which even after a half century

as diagnostic means to detect incipient thrombosis His work further *emphasized that thromboembolism is a complication of medical conditions* as much as it is a postoperative sequela

As the result of careful autopsy studies, a number of workers including Denecke,[7] Clow,[6] Homans,[14] Rossle,[28] Neumann,[21] Putzer,[25] Frykholm[9] and Hunter and his group[16, 17] established that thrombosis usually begins in the deep veins of the calf and even in the plantar veins, and propagates in the direction of the blood stream The *process rarely if ever has its beginning in the large vessels such as the* femoral veins

Another significant step in the advance of thrombosis was made by Ochsner and DeBakey[23] who differentiated intravascular clotting into two classes Phlebothrombosis and thrombophlebitis In the first, the clot is formed independent of inflammation and is not firmly attached to the vessel wall, while in thrombophlebitis the clot is *firmly adherent and has resulted from an inflammatory condition* in the wall of the vein In practice, the border line between the two types is often not sharp and considerable overlapping occurs

## Chapter 5

# VENOUS THROMBOSIS

VENOUS thrombosis and pulmonary embolism present a major problem in both medicine and surgery If judged by the number of papers devoted to it, particularly in current surgical journals, it can be considered as one of the most extensively studied subjects at the present time Even a brief review of the contributions made during the past few years would be beyond the scope of this chapter. The presentation will be strictly limited to the application of the new concepts developed in the study of hemostasis to a consideration of venous thrombosis

### HISTORY

Since few advances can be made without the solid foundation of past experiences and findings, it is well to summarize briefly the history of thrombosis or better of the thrombo-embolic state There is a certain amount of parallelism between the study of thrombosis and hemostasis Though the pursuit has been along different channels, the relation of the one to the other was recognized by the early writers In 1892 Alexander Schmidt[29] wrote "He who busies himself with the critical study of coagulation and assumes the task to establish its cause, strives implicitly at the same time to understand the reason for the permanent fluid state of circulating blood, for it is clear that the moment we fully comprehend the clotting of fibrinogen in its basic relationships, we have also found the answer to the problem why this process does not occur in the living organism " (Author's translation )

The first specific mention of intravascular clotting was made by Petit[24] in 1731 A half century later, John Hunter[15] stated that thrombosis was caused by an inflammation in the wall of a vessel Another sixty years elapsed before the classical studies of Virchow[32] made their appearance To him belongs the credit for the discovery that pulmonary embolism is the result of clots formed in the veins far removed from the lungs To quote his own words "Every time I found a clot in the pulmonary artery, I always succeeded in tracing other

specifically limited to the platelets, needs little discussion Recently much effort has been spent in an attempt to show that under certain conditions, platelets are supposedly more sticky. The measurement of this property is made entirely empirically and under conditions grossly unphysiological. For a comprehensive review of this subject the paper of Moolten and his associates [20] should be consulted Until a procedure is devised which approximates intracorporal conditions more closely, attempts to measure the behavior of platelets must be evaluated with much skepticism

The fourth factor remains, namely, changes in the vessel wall. It can be stated with a fair degree of certainty that no coagulation occurs in the intact vascular system provided there is no break in the endothelium lining. If this statement is accepted, it naturally follows that a change somewhere in the endothelial layer of the venous system is

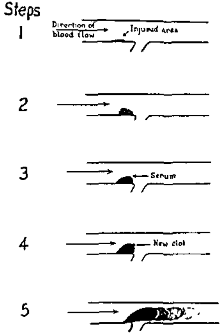



FIG. 4 The probable sequence in the formation of a venous thrombus

Step 1 Injury of vessel wall, adherence of platelets to local area of trauma

Step 2 Disintegration of platelets Formation of a reticulated fibrin clot enmeshing intact platelets

Step 3 Retraction of primary clot thereby expressing a serum rich in nascent thrombin

Step 4 Formation of a new fibrin coagulum attached to the primary thrombus

Step 5 Growth of thrombus by successive formation of new clots, and repeated elaboration of nascent thrombin by clot retraction

## THE MECHANISM OF THROMBOSIS

In a critical analysis of the problem of thrombosis, the four factors postulated by Aschoff serve as a suitable starting point. The first question to be answered is· which is or are primary factors, and which are secondary?

For convenience, changes in circulation will be considered first. It is well recognized that impairment of circulation is frequently followed by thrombosis. No better example can be cited than the cases of thrombosis of the lower extremities which frequently occurred in the older people who spent long periods during the bombing of London in shelters on hard deck chairs.[31] Obviously, impairment of circulation must be an important factor in the development of these "shelter legs" as the condition was called. One may even venture further and call it the precipitating event, but beyond that one cannot go and call the impaired circulation the primary cause. One must not ignore the fact that many other people sat in the same cramped position for equally long periods of time without suffering thrombosis. Since the latter very likely had the same impairment of circulation, it stands to reason that the circulatory factor alone cannot bring about intravascular clotting It might also be pertinent to mention the old observation (probably first made by John Hunter) that blood in a segment of vein separated from the general circulation by double ligature remains fluid It seems fairly clear that a change in circulation does not initiate intravascular clotting, but is an important contributing factor

The second factor, changes in the coagulability of the blood, is the most difficult to discuss critically. As pointed out in Chapter 2, coagulability is an intangible property that one measures empirically in a glass test tube It is doubtful if one could ever obtain a specimen of blood, even during the period when a massive thrombus is forming, that would show a coagulation time of less than 20 minutes provided the blood be carefully collected and transferred to a silicone-coated test tube A blood with such a prolonged coagulation time outside of the body is not likely to be more coagulable when enclosed in a system lined with natural protective endothelium It should also be mentioned that if thromboplastin or thrombin is injected slowly intravenously no massive thrombosis occurs although a relatively slow but drastic drop in fibrinogen takes place as Jurgens and Studer[18] again recently reported In view of these established facts it is difficult to see how a change in coagulability (as defined by the clotting time) can possibly be the primary or initiating cause of thrombosis

The third factor, a change in the blood elements, which can now be

the wall of the vein. If it were not for retraction, the formation of a thrombus 18 inches in length, attached only at a distal base and floating freely in the femoral and extending into the iliac vein, as is not uncommon, could not occur. Actually a sluggish circulation is more conducive to the formation of these long dangling thrombi which easily become massive pulmonary emboli, than a stagnant pool of blood which would tend to clot solidly in the vein and adhere to the vessel wall.

According to this concept, the primary or initiating cause of thrombosis in an alteration in the vessel wall and the immediate contributing factor is the slowing of the circulation. No qualitative change in the platelets needs to be postulated, since under the influence of the first two factors they will begin their participation in the formation of the thrombus. The number of platelets, however, will markedly influence the speed with which thrombin is formed, and even more important, the extent and rate of clot retraction It is interesting that a rise in the platelet count postoperatively is rather common

Since the propagation of the thrombus according to the writer's hypothesis is largely dependent on clot retraction, this property of the coagulum deserves utmost attention Interestingly enough, Hirschboeck and Coffey[12] reported that clot retraction was accelerated in patients with a thrombotic tendency and recently Hirschboeck[11] has published additional data to support the original findings. He has further devised a simple test for clot retraction He observes the coagulation of a drop of blood suspended in castor oil and accurately times the appearance of a serum globule

The four principal factors which influence both the rate and completeness of clot retraction are (1) the number of platelets, (2) the total cell volume, (3) the speed and amount of thrombin formation, and (4) the concentration of fibrinogen

As pointed out in Chapter 1, it has been known since the work of Hayem that clot retraction is dependent upon the number of platelets In figure 5 one can see clearly the direct relationship between the number of platelets and the degree of retraction It might also be mentioned that the speed of retraction is likewise increased as the platelet count rises Significantly too, thrombocytosis has long been recognized as an important factor in thrombosis Thus, a marked increase in the platelet count usually occurs following splenectomy and this is accompanied by a high incidence of thrombosis.

The influence of cell volume on clot retraction is explainable on a mechanical basis Since the cells are non-compressible, the greater their bulk, the less will be the retraction of the clot In figure 6 the relation of clot retraction to the hematocrit reading is illustrated

the first and essential step in the train of events which eventually may culminate in a massive thrombus and a fatal pulmonary embolus Without question, one of the common mistakes has been the implication that absence of gross or at least histologically demonstrable alteration of the vessel wall is necessary for the thrombotic process to start Moolten and his co-workers[20] by an ingenious device of studying the miniscus of an entrapped air bubble in a small vessel have shown that normal endothelium behaves like a non-wettable surface but that a slight disturbance, too small to be seen histologically, may cause the surface to become relatively more wettable, and thus serve as the locus of origin of the thrombus

On the basis of these considerations, a relatively simple explanation of thrombosis can be offered which is illustrated schematically in figure 4 Any small area on the inner surface of the vein may temporarily become abnormal, *i e*, lose its non-wettable property. Like other wettable surfaces it will then adsorb a minute quantity of thrombin to its surface This thrombin may come from the blood itself, or may arise from a slight exudation of thromboplastin from the vessel wall For the immediate discussion, the source of thrombin is immaterial, *but its spread on the vessel wall, perhaps as a molecular layer is important* As soon as platelets strike this surface, they adhere and begin to disintegrate (Step 1) Since these cells, because of their lightness, travel in the center of the stream, few will come in contact with the defective vessel wall unless the circulation is slowed The first unfavorable effect of impaired circulation therefore becomes obvious, the platelets migrate to the periphery and come in contact with the defective vessel wall The platelets which undergo lysis liberate the activator of thromboplastinogen, and the chain reaction (illustrated in figure 2 and explained in Chapter 2) is set off A reticulated fibrin clot is formed (Step 2)

The third step is perhaps the most important key to the problem of thrombosis It is clot retraction On it depends first the growth of the thrombus and secondly the shrinking of the propagating mass which prevents it from staying in contact with the vessel wall The serum expressed from the clot immediately acquires a high concentration of thrombin and unless the serum is immediately washed away by the flowing blood, massive new coagulation will occur locally Apparently the blood flow generally does remove most of the serum along the sides of the clot, but not from the tip as is to be deduced on purely physical grounds Growth of the thrombus occurs, therefore, almost entirely at the tip If it were not for the shrinking of the body of the thrombus, it would press upon the vessel wall, and there is evidence that when this occurs, the clot becomes adherent to

stressed that anemia is an important etiological factor Shaw[10] in discussing a paper on pulmonary embolism read by Glynn in 1924, emphasized the importance of anemia following hemorrhage as an important cause of thromboembolism, and the chairman, Dr Donald, confirmed this view by citing additional data in support Ochsner[22] attributed the effect of anemia in part to a diminution of cardiac tone Recently Hirschboeck[11] again called attention to anemia as a cause of increased clot retraction and a factor in thrombosis Interestingly, massive pulmonary embolism is uncommon or absent in polycythemia although thromboses occur frequently This can readily be explained by the poor clot retraction of such blood

The third factor which influences the speed of clot retraction is the concentration of thrombin. In the experiment presented in figure 7 increasing amounts of thrombin were added to a fixed suspension

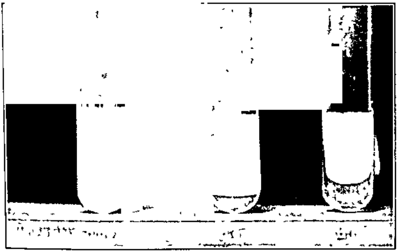
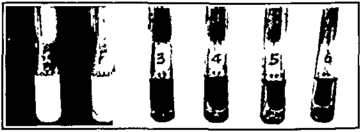


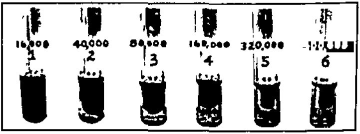
FIG 7 —The effect of the concentration of thrombin on clot retraction
Thrombin was added to a suspension of washed human platelets in a solution of purified human fibrinogen Absorption and adsorption of thrombin by fibrin produces a surface to which platelets adhere, and the metamorphosis, induced by thrombin, which these cells undergo affects the structure of the fibrin reticulum to which the platelets are attached with the result that retraction occurs

of washed platelets in purified fibrinogen It is obvious that clot retraction depends solely on platelets and thrombin This is in accord with the concept of the clotting mechanism presented in figure 2 It may be postulated that thrombin is absorbed by fibrin and that platelets coming in contact with thrombin-coated fibrin strands adhere

Obviously, clot retraction is accentuated by anemia, yet only a few students of the problem of thromboembolism have attempted to correlate anemia with thrombosis While many surgeons instinctively and from experience are aware that the incidence of thrombosis and pulmonary embolism is higher in the anemic patient, few have




½ hour




1 hour

FIG 5 —The quantitative relationship of clot retraction to the platelet count The tubes of native plasma containing increasing number of platelets as marked on the tubes were incubated at 37° C for one hour

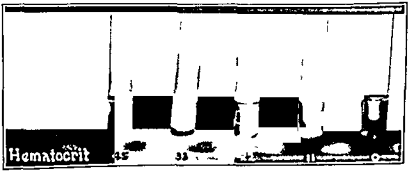



FIG 6 —The influence of cell volume on clot retraction The platelet count was maintained constant but the plasma volume was progressively increased

or consumed during coagulation, it is unlikely that hyperprothrombinemia is an important factor in thrombosis clinically The limiting agent in coagulation nearly always is available thromboplastin. Its production depends primarily on the store of thromboplastinogen and the number of platelets. Unfortunately, it is not known how to control or regulate the latter two agents. The best known means to suppress thrombin production is either to inject heparin intravenously, or to administer Dicumarol Since these two anticoagulants are occupying a prominent place in the therapy of thromboembolism, a discussion of the anticoagulants is warranted

## THE ANTITHROMBINS AND ANTICOAGULANTS AND THEIR RELATION TO THROMBOSIS

**The Normal Serum Antithrombin, Albumin X.**—Much controversy has centered about the anticoagulants and their rôle in the coagulation mechanism and in the maintenance of the fluidity of the blood. Historically, two naturally occurring antithrombins have been recognized, namely heparin and albumin X[24] The latter is generally

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is to be emphasized that the serum antithrombin does not inhibit the action of thrombin on fibrinogen and is therefore not an anticoagulant Heparin, on the contrary, in conjunction with a co-factor acts as a powerful anticoagulant

The relationship of heparin and the normal serum antithrombin are

ing which time fibrin is formed and the thrombin is liberated Both fibrin and fibrinogen have an affinity for thrombin, but since the latter has the stronger attraction, the conversion of fibrinogen to fibrin proceeds until all has been clotted The antithrombin of the serum (albumin X) has such a weak affinity for thrombin that it can not compete with either fibrinogen or fibrin, but in their absence it has the capacity to neutralize large quantities of thrombin Obviously, therefore, the normal antithrombin cannot protect against the clotting of fibrinogen by thrombin It can remove excessive amounts of thrombin but only at a relatively slow rate

**Fibrin, the Physiological Antithrombin.**—It must be recognized that a few milliliters of blood can produce potentially enough throm-

and undergo metamorphosis, since thrombin labilizes these cells The change in the physio-chemical state of the platelet somehow affects the fibrin strand causing it to undergo alterations which results in a shrinking of the fibrin reticulum.

The fourth factor that quantitatively affects clot retraction is the concentration of fibrinogen As already stated, it can be postulated that retraction depends on the adherence of platelets on strands of fibrin, and on the alteration of these cells by thrombin. Therefore the greater the number of platelets per unit strand of fibrin and the higher the concentration of thrombin, the more will be the retraction. It follows, therefore, that if the number of platelets and the concentration of thrombin are kept constant, but the amount of fibrinogen is increased, retraction will become less complete because the ratio of the number of reacting platelets to a strand of fibrin is decreased This is experimentally substantiated by the results presented in figure 8 Since an increase in the concentration of fibrinogen

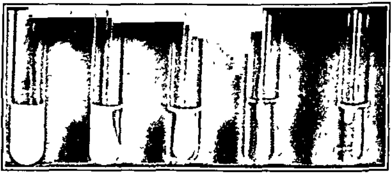



FIG. 8 —The influence of the concentration of fibrinogen on clot retraction The platelet count was 30 000 per cmm and 0 1 cc of thrombin (F S ) was added to each tube

causes clot retraction to become less complete, one can conclude that a rise in the fibrinogen level tends to reduce the thrombotic tendency Some investigators have of course accepted the opposite view because the viscosity of the blood is increased by a rise in the concentration of fibrinogen

Since thrombin quantitatively affects clot retraction, it stands to reason that any increase in the formation of thrombin should tend to produce a thrombotic tendency Theoretically any increase in the prothrombin or thromboplastic factors should favor thrombosis Since prothrombin is normally in excess and is not completely utilized,

theless, heparin with its co-factor Albumin $X_1$ is the most powerful antithrombin known, and has been used widely in the prophylaxis against thrombosis

Heparin actually inhibits clotting in several ways. It is first of all an antiprothrombin, *i e.*, it combines with prothrombin and thereby prevents its conversion to thrombin. Its antithrombic action has already been discussed. Due to these properties heparin is a powerful anticoagulant. It, therefore, stops a thrombotic process expeditiously and prevents the propagation of an intravenous clot Since heparin inactivates thrombin, it preserves the stability of platelets Therefore, in heparinized blood, platelets will not adhere to injured vessel walls, and the formation of new thrombi and potential emboli is prevented.

**Dicumarol.**—Although this compound is not, strictly speaking, an anticoagulant, it is usually placed in this group of agents. It *per se*

effectiveness

Dicumarol reduces the prothrombin of the blood Recent unpublished work of the writer and his associates shows that the free or active prothrombin is decreased first Usually no reduction of the precursor, prothrombinogen, is demonstrable until the drug has been given for several days. The action of Dicumarol is not known It does not destroy prothrombin already formed, but interferes with the mechanism of its synthesis

A plausible explanation for the action of Dicumarol in combating thrombosis can be offered As pointed out repeatedly, thrombin is necessary not only to form a fibrin clot, but also to labilize platelets Without the latter action, platelets fail to bring about the activation of thromboplastinogen or to effect clot retraction If, therefore, the production of thrombin is reduced, the thrombinogenic chain reaction is abnormally slowed and clot retraction is retarded Both these effects are favorable, since they are protection against the formation and growth of a venous thrombus

The lower the prothrombin level is reduced by Dicumarol, the more effective is its prophylactic action against thrombosis According to this hypothesis, even a slight lowering of the prothrombin concentration is already beneficial In practice one strives to attain a level that has the maximum therapeutic action consistent with safety from hemorrhage Both on the basis of theory and from clinical experience a prothrombin level of about 20 per cent is accepted as the ideal

bin to coagulate all of the blood in the body. In the past there has been much speculation how this danger is averted but no satisfactory explanation could be offered until Quick and Favre-Gilly[27] made the important discovery that only a minute amount of prothrombin is converted to thrombin during the period when all of the fibrinogen is changed to fibrin, and that the rapid activation of prothrombin occurs only after the serum is separated from the clot. These observations furnish valuable information concerning the control of coagulation. Initially, a small quantity of thrombin is formed which clots all of the fibrinogen A reticulum of fibrin presenting an enormous surface is formed which quickly and effectively absorbs thrombin. With the immediate removal of this clotting agent, the labilization

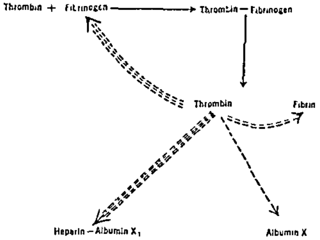



FIG 9 —The interrelationship of the antithrombins
The number of lines in the arrows indicate the relative affinity that the antithrombins have for thrombin

of platelets is markedly retarded, and the chain reaction is held in restraint It is only when the serum separates from the clot, that the intimate contact of serum with the fibrin surface is broken As a result the chain reaction is set off and the production of thrombin occurs rapidly This explains why the serum expressed by the intravascular clot in figure 4 is rich in thrombin, and therefore produces a secondary clot

**Heparin.**—Although this compound is often called the natural anticoagulant, there is no evidence that it has any physiological function relative to hemostasis or thrombosis The organism never secretes heparin into the blood in defense against thrombosis Its employment is probably solely for immunological purposes Never-

theless, heparin with its co-factor Albumin $X_1$ is the most powerful antithrombin known, and has been used widely in the prophylaxis against thrombosis.

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**Dicumarol.**—Although this compound is not, strictly speaking, an anticoagulant, it is usually placed in this group of agents. It *per se* has no effect on coagulation and even the hypoprothrombinemia which it produces is not reflected in the clotting time until the prothrombin is reduced to a level far below that required for therapeutic effectiveness

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The profession owes a debt of gratitude to Dr. Link and his students for the splendid research which made this drug available. For the historical development the reader is referred to Dr. Link's Harvey Lecture.[19] Fortunately, this drug is amazingly safe This is accounted for by the fact that the blood contains an amount of prothrombin far in excess of the requirements for hemostasis. By far the largest number of accidents, *i.e.*, serious hemorrhages attributable to the drug have been caused by inadequate control with proper prothrombin time determinations. Whether Dicumarol will be superseded by better drugs is difficult to predict, but even if it is ultimately replaced, its important rôle in initiating a new approach to the solution of the problem of thrombosis can never be underestimated.

Since the availability of the simple prothrombin time test not only greatly assisted in the isolation of Dicumarol from spoiled sweet clover hay and later made safe its therapeutic employment, one cannot help speculating whether the prothrombin consumption test may not similarly help to bring the thromboplastin factors under control and eventually make possible the utilization of this knowledge for the prevention of thrombosis

***Thrombophlebitis.***—*When a long segment of vein becomes inflamed*, a large area of endothelium becomes abnormal Platelet adhesion will be widespread, with the result that a fibrin clot firmly adherent in its entire course and filling the lumen completely will result Such a clot cannot retract and propagate in the manner of the phlebothrombotic type Pulmonary emboli are, therefore, much less common One of the best examples of pure thrombophlebitis is the idiopathic recurrent type which is commonly called thrombophlebitis migrans This entity illustrates one feature of thrombosis that is often overlooked, namely, the rapidity with which the organism can dissolve a clot This is brought about by fibrinolysin, an enzyme which exists in the plasma in an inactive form Little is known concerning the regulation of this enzyme, but its rôle in thrombosis should not be underestimated It should be pointed out that neither heparin nor Dicumarol have any direct action on the clot already formed, but they assist in preventing an extension of the clot, while the fibrinolysin begins its disintegrating action on the fibrin

There is a certain analogy between thrombosis and lobar pneumonia In both the consolidation is due to a fibrin clot In the normal process, resolution is effected by fibrinolysin and little or no residual damage occurs to the endothelial lining If, however, the process is delayed, infiltration of the fibrin clot by connective tissue sets in, and organization occurs In the lung this leads to fibrosis

while in the veins the lumen becomes markedly narrowed or totally occluded In lobar pneumonia and other inflammatory processes, the deposition of fibrin may be a part of the immunological response. If this be so, one should not overlook the probability that the massive intravascular clotting in thrombophlebitis may perhaps also be purposeful even though the harmful effects may be far more prominent. Some of the chief points of differences between phlebothrombosis and thrombophlebitis may be outlined as follows.

| | *Thrombophlebitis* | *Phlebothrombosis* |
|---|---|---|
| Reason for being | Purposeful | Accidental |
| Primary cause | Alteration in vessel wall, usually due to inflammation | Alteration in vessel wall due often to trivial causes such as anoxia, pressure externally spasm etc |
| Location | Nearly any region of the body | Almost always in the lower extremities |
| Site of origin | In any vein irrespective of size or location | Usually in the plantar or other small veins of the lower extremities |
| Contributary factors | | |
| Venous stasis | Little influence | Marked influence |
| Thrombocytosis | Not significant | Significant |
| Anemia | Without much effect | Predisposing |
| Probable mode whereby thrombus is propagated | Extension of inflammation of vessel wall | Clot retraction |
| Nature of thrombus | Firmly attached to vessel wall | Freely floating in blood stream with few attachments to vessel wall |
| Local physical signs | Pain swelling tenderness and elevated temperature | Mild or no signs |
| Sequelæ | Occlusion of lumen of vein Pulmonary emboli uncommon unless thrombus is suppurative | Pulmonary emboli |

## TREATMENT

The key to the treatment of thrombosis is prevention Reliance on any one agent or approach is not enough In a program of prophylaxis, all the known factors must be considered Since an alteration in the vessel wall is the primary cause of thrombosis, all efforts must be directed to maintain the health of the vessels, particularly of

the lower extremities. Since anoxia is a likely cause of a temporary change in the vessel wall, stasis should not be allowed to occur. Even seemingly trivial factors such as chilling, cramped position, and smoking should not be dismissed lightly.

While only indirect means are available for maintaining the normal physiological state of the blood vessels, the second factor, circulation, can be attacked directly. Most of the measures have been repeatedly discussed in the current literature. For a comprehensive review, the paper of Ochsner[22] is particularly to be recommended. The importance of foot and leg exercise, the avoidance of posture that favors venous stasis (especially Fowler's position), the employment of loose abdominal binders, and deep breathing to increase the negative pressure of the thorax thereby aiding the emptying of the large veins are all important means to combat sluggish circulation in the lower extremities The condition of the heart should not be overlooked. If in the opinion of the physician, digitalis, quinidine or theobromine is indicated, these drugs should be given without hesitation. The uncritical claims that these substances influence the coagulation reactions can be set aside until more convincing experimental and clinical data are offered

No practical means are available to influence the platelets, but since their action is intimately related to coagulation, regulation of the latter is usually adequate for taking care of the platelet factor As already discussed, the coagulability and the thrombinogenesis of the blood is suppressed by both heparin and Dicumarol The efficacy of these drugs to reduce the incidence of thrombosis and fatal pulmonary embolism has been repeatedly reported As examples of several outstanding reports the papers of Barker and his associates,[2] Bauer[3] and Zilliacus[34] may be mentioned

Correction of anemia as a prophylactic measure against thrombosis should be triply underscored The significant effect anemia has on clot retraction has been discussed and the probable rôle of retraction in the propagation of the clot is illustrated in figure 4 Hemorrhage is particularly apt to predispose the patient to thrombosis, for not only does it cause anemia but it often is followed by a marked thrombocytosis A transfusion to correct the anemia should be regarded as an important means to protect the patient against thromboembolism

No attempt will be made to discuss the pros and cons of vein ligation The surgeon's judgment should be paramount, but that judgment should be tempered by a full appreciation of the possibilities of the anticoagulants Much has been accomplished by both those who favor and those who oppose ligation as a prophylactic measure against

pulmonary embolism. Both sides agree that neither possesses the means to determine which patient will suddenly and without warning die of a massive pulmonary embolism before either anticoagulants can be given, or a femoral vein can be ligated. When the enigma regarding the events that lead to this catastrophy has been completely solved, the controversy of ligation versus anticoagulants will become merely of historic interest.

**Recapitulation.**—As the result of the studies of many investigators, it is fairly well established that the majority of thromboses which result in pulmonary embolism have their origin in the deep veins of the calf or in the plantar veins. The principal factors in thrombosis are an alteration in the vessel wall, slowing of the circulation, increase in the number of circulating platelets and a change in the coagulability of the blood. It is postulated that the thrombus is initiated by a local change in the endothelium of a small vein which acts as a focus for the accumulation and adherence of platelets The disintegration of platelets brings about the formation of thrombin which produces a *fibrin clot. When clot retraction sets in, a serum is expressed in* which a high generation of thrombin occurs, thus bringing about the formation of a new clot attached to the original thrombus By repeated retractions and the production of new clots by the extruded serum, the thrombus is propagated Clot retraction plays an important rôle in thrombosis, since in the unretracted clot the fibrin with its tremendous surface, because of its dispersed state in the reticulum, removes thrombin promptly and effectively In the prophylaxis against thrombosis, the avoidance of venous stasis in the lower extremities is essential The correction of an existing anemia is important since the speed and extent of clot retraction increases as the hematocrit reading decreases The two drugs effective in the prevention of thrombosis are heparin and Dicumarol The first is a powerful antithrombin It inactivates thrombin, thereby stopping coagulation and further growth of the thrombus Dicumarol pro-

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# Part II. Laboratory Methods

## INTRODUCTION

The diagnosis, management of therapy and prognosis of the hemorrhagic diseases depends largely on laboratory methods The clinician should be familiar with the various tests which are contributing to the understanding and control of abnormal bleeding, and the laboratory worker should have available explicit directions to carry out these tests The writer has selected the methods which he considers the most valuable for the clinical study of the hemorrhagic diseases In outlining the various procedures, he has had the advantage of long and intensive experience with them Every test described has been repeatedly checked by the writer and his associates both on normal and pathological bloods No attempt is made to present a historical background for these tests nor to compare them with similar procedures developed by other investigators Proper credit for priority however is given

It is to be emphasized that meticulous attention to details is a primary requirement for accurate results It is equally important that the details should be correct If one should follow, for instance, the directions for the one-stage method for the determination of prothrombin as given in a recent book, in which interestingly enough 5 per cent of the entire content of the volume is devoted to this test, one could not possibly obtain precise and correct values because of a few seemingly minor deviations from the original procedure It is most disconcerting that the originator of a test has no control over his own invention Inferior modifications are readily and uncritically accepted and promulgated Research programs national in scope in which his test is an integral part are set up without any effort being made to consult him Yet withal he remains the recipient of all the hostile criticisms

A common fallacy is to conclude that a method lacks accuracy because it is simple Often such a procedure, to the contrary, may be far more accurate than a more complex test consisting of several steps. Unfortunately, however, there is a tendency to permit carelessness to creep in when a test appears simple Too often workers of limited experience and ability who will not attempt doing a complex pro-

cedure will have little hesitancy trying their luck with a seemingly simple test. It is easy to blame ones' unsatisfactory results on a sloppy method; but on close scrutiny it is often found that the adjective is more applicable to the technician In the methods selected, accuracy was never sacrificed for simplicity, but judgment was exercised in deciding what the limits of accuracy should be The methods as outlined meet the clinical demands as based on present day knowledge Any well-trained laboratory worker should be able to carry out any one of the tests by following the directions outlined.

While the procedures are selected mainly for their clinical utility, *this does not gainsay that they are equally suitable for research. It* was through these tests that the labile factor was discovered, that the existence of thromboplastinogen in plasma was established and its lack in hemophilia pointed out By means of the prothrombin consumption test, the function of platelets in activating thromboplastinogen was demonstrated, and for the first time it was shown that a true coagulation defect is present in thrombocytopenia These tests made it possible to establish that prothrombin in human plasma exists partly free and partly in a precursor state, a concept which makes it possible to differentiate two types of congenital hypoprothrombinemia

It has become almost a fetish with certain investigators that systems of partly purified reagents are the only legitimate means for doing research in coagulation While this approach may ultimately produce valuable data, it has in the past led to several serious errors In evaluating the results obtained from the study of artificial systems, one should carefully differentiate between purified and pure agents Blood is an intricate and delicately balanced system in which numerous factors interact The moment blood is withdrawn changes are introduced and the more the blood is manipulated, the greater will be the alteration in the balance and equilibrium of its true physiological state One must be particularly cautious in drawing conclusions from results obtained on blood collected in a slaughter house because by the time such blood reaches the laboratory, irreversible chemical changes can and do occur These statements are in no way intended to be derogatory of the efforts to obtain the various clotting *factors in pure form The availability of heparin, thrombin,* fibrinogen, Dicumarol and other agents in pure or purified form has been immeasurably helpful in research But it is unfortunately becoming necessary to combat actively the erroneous notion that the

that appeared absurd or foolhardy to the more sagacious scientist have succeeded at times when astute plans of research failed. It is well to remember that it isn't enough merely to transfer blood to a test tube; one must also carry with it an intuitive feeling for physiological reactions and an insight into the scheme of body function.

## GENERAL COMMENTS AND SUGGESTIONS

The routine tests needed for the study of the hemorrhagic diseases are relatively simple, and do not require elaborate, complicated or expensive equipment Nevertheless to carry out these procedures successfully and accurately, fundamental training in laboratory technique and strict adherence to a few basic rules are primary requisites

The control of temperature in coagulation tests should be mentioned first, since most of the reactions measured are significantly influenced by variations in temperature. One must emphatically condemn the practice of doing tests at room temperature Since many laboratories have a temperature range from 18 to 32° C, it is easy to see how ridiculous, unscientific and meaningless the term, room temperature, is Most of the tests described are carried out at 37°–38° C. since it seems logical to study the clotting reactions at body temperature In some tests, however, a temperature of 25° C is selected, which can be considered the equivalent of a constant room temperature A constant temperature water bath as described in Test 1, should be part of the standard laboratory equipment The ordinary vacuum bottle serves as a convenient and inexpensive portable water bath which is particularly suitable for incubating solutions and mixtures

Standardization of glassware likewise is important for satisfactory results Borosilicate glassware (Pyrex or Kimble) is recommended The 13 × 100 mm test tube is used routinely in all tests unless a a different size is definitely specified This test tube is convenient to handle and is easily cleaned The 1 cc serological pipette graduated in tenths and hundredths of a cc is likewise standard equipment Since its length makes it cumbersome, it is shortened to 170 mm by cutting The end is fire polished The 0 2 and 0 1 cc serological pipettes are also employed in many tests By means of a mechanical pipette filler marketed under the name Pipette Suction Apparatus (Clay-Adams A2473) quantities of 0 01 cc and less can be measured and transferred from a 0 1 cc pipette with a high degree of precision

The proper cleaning of glassware requires particular emphasis because several clotting factors are significantly affected by surface contact An agent like thrombin is adsorbed by glass and is not re-

moved by mere rinsing as the following experiment clearly illustrates. One cc of thrombin was transferred to a test tube and after 1 minute it was poured out. The tube was drained and then thoroughly rinsed 3 times with saline solution It was calculated that whatever thrombin remained in the test bube was diluted $3 \times 10.^7$ Nevertheless when 1 cc. of hemophilic blood, which clotted in 50 minutes in a control test tube rinsed only with saline, was transferred to the test tube that had contained thrombin, a solid clot formed in 8 minutes Such a finding can only be explained by assuming that the glass surface adsorbed thrombin and held it so firmly that repeated rinsing did not remove it. This simple experiment emphasizes the necessity for scrupulously clean glassware.

Glassware is routinely cleaned as follows. Test tubes are rinsed with tap water immediately after use, then soaked in soap solution or a detergent made for chemical ware, and finally washed on a revolving test tube brush driven by a water motor. In lieu of the latter, thorough scrubbing with a test tube brush by hand is necessary. The test tubes are repeatedly rinsed with hot tap water, finally with distilled water, placed in a wire basket to drain and are air dried. *Chromic acid cleaning fluid is not used for cleaning test tubes.* Badly scratched test tubes are discarded

Pipettes are best cleaned with chromic acid mixture The acid must be completely removed by repeated rinsing with tap and finally distilled water

*Coating of Glassware with Silicone.*—Glassware is thoroughly cleaned, rinsed and dried One volume of silicone (methyl-chlorosilane, marketed under the trade name Dri-Film No. 9987, General Electric Co ) is mixed with 3 volumes of toluene This mixture is poured into the container to be coated After the solution has been in contact with every part of the surface, it is poured out, and the container drained for 30 minutes It is then thoroughly rinsed in warm running water, and air dried The surface is polished with a nylon cloth until smooth It is again treated with the silicone-toluene mixture. For meticulous work, six coats of silicone are applied After each the surface is polished with a nylon cloth except after the final coating The coated glassware is protected from dust After the glassware has been used, it is cleaned with a detergent, rinsed, dried, polished with a nylon cloth and again given one coat of silicone To coat needles, the silicone-toluene mixture is passed through, then the excess is blown out by compressed air The needle is immediately dropped into a dilute ammonium hydroxide solution to prevent contact with the free hydrochloric acid which is liberated when methyl-chlorosilane is hydrolyzed The bore of the needle is polished with a nylon bristle As with glassware six coatings are applied

Silicone-coated ware must be kept separate from the other glassware. The brushes used to clean silicone-coated glass should be reserved exclusively for that purpose. Silicone-coated glassware cannot be cleaned with chromic-sulfuric acid cleaning solution. The complete removal of silicone is well-nigh impossible, therefore any apparatus treated with silicone cannot be reclaimed

*Collection of Blood.*—With few exceptions, the tests are carried out on blood collected by venipuncture. If trouble is encountered in entering a vein, it is advisable to start again using a fresh needle. In infants the scalp veins often can be used advantageously for obtaining blood, and sometimes the arm veins are suitable Routinely, nine volumes of blood are mixed with one volume of the anticoagulant solution (sodium oxalate or citrate) It is to be remembered that 1 cc. of such plasma contains only approximately 0 9 cc. true plasma Native plasma can readily be obtained by collecting and keeping blood in silicone-coated glassware On slow centrifugation (800 r p m ) a platelet-rich plasma is obtained, whereas at 8,000 r p m a platelet-poor plasma results.

*Stock Solutions* —Physiological saline solution 8.5 gm of sodium chloride are dissolved in 1 liter of distilled water

Sodium oxalate 0 1 M.—13 4 gm of c p sodium oxalate are dissolved in distilled water and diluted to 1 liter It is kept in the refrigerator

Sodium citrate 0.2 M —58 8 gm of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) are dissolved in distilled water and diluted to 1 liter.

Sodium citrate 0 1 M. One volume of the 0 2 M solution is diluted with an equal volume of distilled water Both 0 1 M and 0 2 M sodium citrate solutions should be kept in the refrigerator

Calcium chloride 0 1 M —11 1 gm of andydrous calcium chloride are dissolved and diluted to 1 liter.

Calcium chloride 0 02 M —20 cc of 0 1 M calcium chloride is diluted with 80 cc of distilled water.

Calcium chloride 0 01 M.—10 cc of 0 1 M. calcium chloride is diluted with 90 cc of distilled water

*Special Equipment* —The instruments listed are being used in the writer's laboratory This does not imply that they are necessarily superior to others on the market, but they are entirely satisfactory.

Centrifuges

1) International Clinical Centrifuge.
2) Angle Centrifuge Sorvall Type SS.
3) Angle Centrifuge Sorvall Type XL.

Beckman pH Meter, Model G.

Coleman Spectrophotometer, Junior Model 6A

Constant Water Bath Technicon

## Test 1

### THE COAGULATION TIME

*Definition* — The coagulation time of blood is a measure of the time required for sufficient fibrin to form in a mass of extracorporal blood, collected and kept under specific conditions, to convert it from a free flowing to a gelatinous state.

*Principle* — To determine the intrinsic coagulative power of the blood, collection must be made without admixture of tissue juice, and the test should be carried out on a fixed volume of blood in a glass test tube of a fixed diameter at a constant temperature It is a measure of the composite factors that participate in the formation of thrombin and the conversion of fibrinogen by thrombin to fibrin under standardized conditions

*Equipment*

Syringe - 5 or 10 cc coated with silicone (Dri-Film)
No 20 needle coated with silicone
Test tubes — 13 × 100 mm. with an internal diameter of exactly 11 mm.
Water bath kept at 37½° C

*Procedure* — Blood is obtained by venipuncture. A tourniquet is applied just prior to the puncture If the vein is not entered immediately and without trauma, the operation is repeated using a new needle and a different vein The needle is removed from the syringe and 1 cc of blood transferred into each of two clean, dry test tubes, *care being taken to avoid foaming and bubbles* *The tubes are placed* in the water bath, and tilted gently every 30 seconds If clotting does not occur in 10 minutes, the tubes are tilted every 60 seconds. If the clotting time is beyond 20 minutes, it is sufficient to examine the tubes only every 5 minutes For doing the coagulation at the bed side, a vacuum bottle fitted with a cork having a hole in which a test tube can be inserted, serves as a convenient water bath The range for the coagulation time of normal blood is 5 to 10 minutes

To measure the incipient coagulation time, a glass rod is put in a test tube containing 1 cc of blood The rod is gently withdrawn every 30 seconds. A fine thread of fibrin marks the beginning of clotting This occurs in 3 to 4 minutes in normal blood and is usually followed by a solid clot 2 to 6 minutes later In hemophilic blood, the beginning clot may occur in 10 minutes but the formation of a solid clot may require 1 to 2 hours

*Application and Interpretation* —The clotting time serves two important uses It is a screening test in the diagnosis of hemorrhagic diseases and it is the most practical means for controlling heparin therapy.

A prolonged clotting time is always significant, whereas a normal value gives no assurance whatsoever that the patient has normal hemostasis If blood fails to clot, afibrinogenemia is the most likely diagnosis. The most common disease in which clotting is much prolonged is hemophilia The presence of approximately 20 gammas of heparin per cc. of human blood will prolong the clotting time to approximately 30 minutes

[illegible]

test are far more sensitive in detecting deficiencies in the coagulation mechanism

It should be emphasized that a minute amount of tissue juice which may inadvertently gain entrance into a specimen of blood can reduce a clotting time of 2 hours to 8 minutes or less Due to the great difficulty of obtaining blood from a newborn infant, the true prolonged coagulation time in a hemophilic baby is often missed

There is no longer any justification for doing routine coagulation times on capillary blood since a normal value merely gives the surgeon a false sense of security One of the few excuses for doing clotting times on capillary blood is in the control of heparin therapy when it is difficult to obtain venous blood

For a comprehensive discussion of the uses and limitations of the coagulation time, the article of Quick and his associates should be consulted

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## Test 2

## THE COAGULATION TIME OF RECALCIFIED PLASMA

*Definition.*—The coagulation time of recalcified plasma is the time required for a fibrin clot to appear in decalcified plasma, obtained from blood mixed with a fixed amount of sodium oxalate, to which a standard quantity of calcium chloride has been added.

*Principle.*—On adding sodium oxalate to blood, calcium is removed as an insoluble salt and coagulation is thereby prevented. On re-adding calcium, the coagulation reaction is re-initiated. In normal blood many platelets are disrupted before decalcification is complete, whereas in hemophilic blood the platelets remain mostly intact. Therefore, on high centrifugation of oxalated hemophilic blood many platelets are removed, and the resulting coagulation time of the highly centrifuged plasma is prolonged.

*Equipment and Reagents:*

| | |
|---|---|
| Same as Test 1, plus | 0 1 M sodium oxalate |
| Centrifuges | 0 01 M. calcium chloride. |

*Procedure* —Blood is collected with a silicone coated syringe and needle. To each of two glass test tubes containing 0.2 cc. of 0.1 M. sodium oxalate, 1 8 cc of blood are added After mixing, one test tube is centrifuged for 5 minutes at 1,000 r p m. while the second is spun in an angle centrifuge at 3,000 r p m for 5 minutes

TABLE 8 —THE CLOTTING TIME OF RECALCIFIED OXALATED PLASMA AS A TEST FOR HEMOPHILIA

| | | | *Coagulation time* | |
|---|---|---|---|---|
| | | | *Recalcified plasma* | |
| | | *Lee White method* min | *After low centrifugation* sec | *After high centrifugation* sec |
| Normal | I | 6 | 90 | 105 |
| | II | 6 | 125 | 145 |
| | III | 5 | 105 | 130 |
| Hemophilia | I | 38 | 180 | 630*–1080† |
| | II | 22 | 165*–195† | 480*–660† |
| | III | | 180 | 330 |
| | IV | 40 | 165*–200† | 300*–600† |
| | V | 73 | 540 | 900 |
| | | | 180‡ | 180‡ |

* Beginning of coagulation.
† Formation of a firm clot
‡ After standing 6 hours

The clotting time of the two plasmas is determined by mixing 0.1 cc. of plasma with 0.2 cc. of 0.01 M. calcium chloride. The calcium chloride is forcibly blown with the plasma, and the period from that moment to the formation of a fibrin film is carefully timed with a stop watch. Normal human plasma has a clotting time of 80 to 120 seconds for the low centrifuged plasma and 105 to 150 seconds for the high centrifuged specimen. For hemophiliacs both low and high centrifuged plasma have a prolonged value and the latter is usually twice as long as the former.

*Application and Interpretation.*—The procedure has been useful in the diagnosis of hemophilia as shown in table 8.[1] It is not specific, however, for this disease. With the introduction of the prothrombin consumption test, the procedure has become dispensable for diagnostic purposes, but occasionally is useful.

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## Test 3

### BLEEDING TIME

*Definition* — The time required for bleeding to cease after a stab wound of the skin not over 4 mm deep is designated as the bleeding time.

*Principle* — The bleeding time is a measure of the ability of the skin capillaries to stop bleeding after a standardized stab wound. The mechanism that brings about this response remains undetermined. Endothelial adhesiveness *after injury, reflex contraction,* platelet thrombi and release of thrombotonin from labilized platelets may be factors in closing or obliterating bleeding capillaries

*Equipment*

1. A spring lancet with an adjustable blade. The cutting depth of the blade should be fixed at 2 mm.
2. Ordinary filter paper

*Procedure* — The lobe of the ear is cleansed with alcohol, and a cut 2 mm deep is made with a spring lancet Every 30 seconds, the droplet of blood is blotted with the filter paper Normally, bleeding stops in 3 minutes or less The results obtained on a normal subject and on a patient with thrombocytopenic purpura are recorded in figure 10

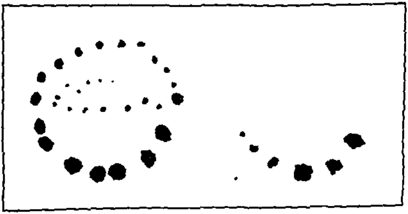


FIG. 10 — The bleeding time test
The filter paper to the right is the record of a normal subject while that on the left of a patient with thrombocytopenic purpura The bleeding surface was blotted every *30 seconds*

When the result is equivocal, *i e*, slightly more than 3 minutes, the test should be repeated on the other lobe of the ear. If bleeding continues after 30 minutes, it is stopped by pressure and by a coating of collodion. Occasionally it is necessary to apply a mixture of thromboplastin and epinephrin to control the bleeding

*Application and Interpretation.*—The bleeding time test is exceedingly important in the diagnosis of hemorrhagic diseases In pseudo-hemophilia it is usually the only positive laboratory finding It is commonly prolonged in thrombocytopenic purpura, but it is not directly related to the platelet count A normal bleeding time is not infrequently observed with a low platelet count In non-thrombocytopenic purpura, the bleeding time is normal, and it is not prolonged in hemophilia, nor in hypoprothrombinemia. If, therefore, the test is abnormally long in either condition, one can conclude that a secondary dysfunction has been superimposed on the primary defect

The bleeding time is not a measure of capillary fragility In scurvy, the test is normal Frequently a prolonged bleeding time is observed when the tourniquet test is positive, but this association is co-incidental In pseudo-hemophilia the tourniquet test is often negative, whereas it is not unusual to find a normal bleeding time in thrombocytopenic purpura, but a strongly positive tourniquet test

In certain patients the bleeding time is sufficiently important and informative to warrant having the physician do the test instead of entrusting it to an indifferent and unskilled technician Much can be learned by observing the reaction of the skin to the cut

## Test 4

### PLATELET COUNT

*Principle* – Platelets in the absence of thrombin remain discrete, show no tendency to agglutinate and do not adhere to a foreign surface By drawing blood into a silicone syringe and mixing it immediately with sodium citrate, no formation of thrombin is permitted and the platelets can be counted in an ordinary counting chamber in the same manner as are erythrocytes

*Equipment and Reagents*·

Microscope. Red cell pipette.
Counting chamber Glass dish with a cover and a small sponge
Silicone coated test tube, 10 × 75 mm, with calibration mark for 1 5 cc.
Silicone coated syringe and needle
Diluting fluid

| | |
|---|---|
| Sodium citrate | 3 8 gm. |
| Formaldehyde (40%) | 0 2 cc |
| Distilled water | 100 0 cc. |

This solution is the same as that of Rees-Ecker, except that the dye, brilliant cresyl blue, is omitted The reagent must be filtered just prior to using it

*Procedure* – Blood is collected in a silicone-coated syringe. Exactly 1 cc is transferred to a small silicone-coated test tube, immersed in an ice bath, containing 0 5 cc of the sodium citrate diluting fluid The test tube is covered with parafilm (a paraffin coated paper) and inverted several times to effect mixing The citrated blood is drawn up to the 0 5 mark of a red blood cell pipette which is then filled to the 101 mark with the diluting fluid The pipette is shaken either by hand or in a mechanical shaker for 5 minutes. Both sides of the counting chamber are filled with the diluted blood The platelets are allowed to settle by placing the counting chamber for 15 minutes in a flat glass dish provided with a cover To prevent drying a small sponge soaked with water is placed in the dish

The platelets are counted using the 4 mm objective of the microscope The platelets are recognized as highly refractile bodies which are usually about one-fourth as large as an erythrocyte but may oc-

casionally be very much smaller Continuous adjusting of the focus of the microscope is necessary to detect all the platelets in the field. The platelets in 2 separate sq mm fields are counted. To calculate, the count in 1 sq mm is multiplied by 2,000 and then by ½ to correct for the initial dilution. The platelets may vary normally from 150,000 to 500,000 A red cell count may be made on the same preparation after the platelet count is completed

The method as outlined assures a platelet count equally as accurate as a carefully performed erythrocyte count. Routinely, platelets are counted by drawing capillary blood directly into a red cell pipette The procedure can be improved by first drawing diluting fluid to the 0 5 mark and then drawing blood to this mark, after which the pipette is filled to the 101 mark.

When the platelet count is exceedingly low, it may be helpful to centrifuge the citrated blood after chilling in an ice bath for 5 minutes at 800 r p m and do the count on the plasma Quick and his associates[1] have found that the platelet count in plasma obtained by slow centrifugation is approximately double that of whole blood

*Application and Interpretation* —For a discussion of the significance of the platelet count in hemostasis the section on thrombocytopenia in Chapter 3 should be consulted If platelets are deficient, the plasma thromboplastinogen is inadequately activated, and little prothrombin is converted to thrombin This quantitative deficiency of thrombin causes impaired hemostasis which the clotting time fails to disclose since it is almost always normal in thrombocytopenia

The platelet count of peripheral blood may not always be a true reflection of the production of platelets by bone marrow Doan[2] has noted that after severe bone marrow depression and a marked thrombocytopenia there is a considerable lag between the restoration of the platelets in peripheral blood and the appearance of new functioning megakaryocytes Only after the production of platelets exceeds the rate of destruction, can there be an accumulation of the blood

The platelet count has prognostic significance in acute leukemia Frequently, the platelet count rises during a remission induced by folic acid antagonists such as 4-amino, pteroyl-glutamic acid (aminopterin) Interestingly, the production of platelets is depressed in pernicious anemia, and is stimulated by liver extracts

The platelet count *per se* yields no information concerning the physiological activity of these cells Lack of platelet function has been designated thrombasthenia Usually the dysfunction implied is failure to cause clot retraction Since it is dubious whether clot retraction has any hemostatic purpose, it seems better perhaps to

reserve this term for one of the important functions of the platelets The two primary functions are the activation of plasma thromboplastinogen and the liberation of a vasoconstrictor. The possible assay of the platelets' potency in activating thromboplastinogen will be discussed under the "Prothrombin Consumption Test."

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## Test 5

### CLOT RETRACTION

*Definition* —Clot retraction is the spontaneous shrinking of a blood coagulum with the expression of a clear serum.

*Principle.*—When blood clots, a fibrin reticulum is formed which enmeshes the cellular constitutents including the platelets. The fibrin strands absorb thrombin and gradually become coated with this agent, thereby presenting a surface to which platelets adhere. Under the influence of thrombin, the platelets undergo metamorphosis which results in physio-chemical changes that affect, perhaps in a passive manner, the fibrin strands to which platelets are attached, causing them to twist, shorten and contract

The degree and speed of clot retraction is dependent upon three intrinsic factors the number of platelets, the quantity of thrombin and the concentraction of fibrinogen, and two extrinsic factors the nature of the walls of the container, and cell volume. Increase in either platelets or thrombin augment retraction whereas the higher the concentration of fibrinogen the less the contraction. Certain surfaces such as collodion have such a great affinity for fibrin that they will not permit clot retraction even of normal blood The greater the cell volume, the less the contraction In determining the retraction of plasma, the cell volume as a factor is eliminated The mechanics of clot retraction has been critically discussed recently [1,2]

*Equipment*·

Same as for Test 1, plus
Clinical centrifuge.
Test tube filled with castor oil U.S P

*Procedure* —Clot retraction, whole blood Two cc. of blood obtained by venipuncture are transferred to a standard test tube which is then placed in a water bath at 37° C Normal blood shows retraction in 30 to 60 minutes

*Clot Retraction, Native Plasma* [3]—Blood collected by venipuncture is transferred to a silicone-coated test tube which is immersed in an ice bath After the blood is chilled, it is centrifuged at 800 r.p m for 10 minutes Usually 5 to 6 cc of blood is sufficient to obtain 2 cc of plasma This amount of plasma is transferred to a standard test tube which is placed in a water bath at 37° C. The tube is examined every minute to detect the beginning of clot retraction. Plasma with

a normal platelet count will generally show clot retraction in 8 to 12 minutes

*Hirschboeck's Clot Retraction Test:*[4]* "The skin of the finger tip is cleansed with alcohol and a puncture 3 mm deep is made with a No 11 Bard-Parker blade with a cork guard set 3 mm. from the tip The time is recorded when the skin puncture is made. Two samples of blood (20 c mm. each) are drawn as quickly as possible into Sahli hemoglobinometer pipettes. The same pipette may be used for each sample if the blood flows freely and the aspiration is done quickly. Each sample of blood is suspended in a separate test tube filled with castor oil, U S P, in the following manner. The blood is expelled from the pipette as a large single drop which is 'planted' on the center of the oil surface by touching the tip of the pipette to the oil The drop will settle into the oil and hang by surface attraction to the meniscus Castor oil was selected because its specific gravity at room temperature is almost the same as the specific gravity of blood The tubes are stoppered to prevent evaporation This procedure should not take longer than 20 seconds Two samples are used for purposes of control If the clot retraction time is not equal or within three minutes of being equal in both tubes, the test is repeated This is seldom necessary The tubes are placed in a rack at room temperature and are observed at the end of 10, 15, and 20 minute periods The end point of coagulation is not determined The beginning of clot retraction is established when a visible dimpling of the clot surface with extrusion of a tiny droplet of serum occurs It is usually readily seen because of the highly refractile oil-blood interface This is the end point of the test The droplet of serum enlarges until clot retraction is complete It was found that normal individuals have a clot retraction time of 20 minutes or longer, the average being 33 1 minutes Clot retraction times longer than 50 minutes were observed only in thrombocytopenia and erythremia and in persons with a coagulation defect The clot retraction time may fluctuate during the course of the day in some individuals"

*Application and Interpretation* Clot retraction is essentially an indirect measure of the platelet count in most instances Since the measurement of clot retraction furnishes no information which cannot be obtained more accurately by other means, all the various procedures developed to determine the ratio of the volume of serum to that of the clot are of limited value either scientifically or clinically.

Since it is becoming exceedingly doubtful that the fibrin clot serves hemostatically as a mechanical plug, the speculation that a non-

* The writer has had no opportunity to test Hirschboeck's clot retraction test clinically, but he feels that it should be given a thorough clinical trial.

retractile clot, such as is formed in thrombocytopenic blood, is faulty in stopping bleeding because of its friable, fragile and non-adhesive character, is no longer convincing. Likewise, the belief that clot retraction serves in drawing together the walls of the injured vessels can be summarily dismissed. The retractile force in the clot is far too weak to be effective for such a purpose. It becomes very doubtful, therefore, that clot retraction influences hemostasis directly. The information whether blood shows retraction or not adds little to diagnosis or prognosis.

Since experimental and clinical evidence points strongly to clot retraction as a key factor in venous thrombosis,[5] a procedure to detect an abnormal hyperretractility may prove to become clinically valuable, as the findings of Hirschboeck suggest. Such a test complemented with a platelet count, a hematocrit reading, and a prothrombin consumption test may materially aid in detecting the true or potential thrombotic state of the blood.

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## Test 6

### THE TOURNIQUET TEST

*Definition.*—The tourniquet test, also known as the Rumple-Leed, Hess or capillary fragility test, and as *le signe du Lacet* in France, is a measure of capillary fragility and hyperpermeability. The test consists in obstructing venous blood flow and observing the number of petechiæ formed in a unit area of skin distal to the obstruction

*Principle* —Capillary fragility is essentially a hyperpermeability which allows the escape of the cellular elements of the blood, causing petechiæ and ecchymoses The specific cause is not known. Theoretically it may be due to an anatomical defect, a toxin or the lack of a factor essential for capillary tonicity In the acute stage, the petechiæ and ecchymoses appear spontaneously and the condition is recognized as purpura By obstructing the venous blood flow, the intracapillary pressure is raised sufficiently to disclose a latent hyperpermeability, shown by the appearance of petechiæ below the level of the obstruction Obviously, a tourniquet test need not be done when showers of petechiæ appear spontaneously

*Equipment*·

Sphygmomanometer and a stethoscope

*Procedure* —The blood pressure cuff is placed on the upper arm, and the blood pressure taken A pressure midway between the diastolic and systolic is maintained for 5 minutes A circle 5 cm in diameter is drawn on the flexor surface of the forearm a little below the bend of the elbow The number of petechiæ formed in 15 minutes following the release of pressure are counted and recorded A count above 5 can be regarded as positive

*Application and Interpretation* —The interpretation and evaluation of a positive tourniquet test is difficult While a positive test is often accompanied by a prolonged bleeding time and a thrombocytopenia, there does not appear to be any direct relationship The tourniquet test is often positive when the platelet count is normal, and conversely no abnormal capillary permeability may be demonstrable in in rather severe thrombocytopenia The relation to the bleeding time is equally inconsistent It seems fairly certain that the bleeding time is essentially a measure of the capillaries' response to injury which may or may not be related to hyperpermeability Thus, in pseudo-hemophilia the bleeding time is prolonged, but the tourniquet

test is often negative In non-thrombocytopenic purpura, the bleeding time is normal but the tourniquet test is usually positive.

The tourniquet test is of limited value in the study of hemorrhagic diseases. It is positive in a great variety of conditions; but in many of these, hemorrhage is either a minor factor or is entirely absent. Even in scurvy, the test is not always positive and often is negative in subclinical vitamin C deficiency. How often capillary fragility and hyperpermeability are due to vitamin P deficiency is difficult to estimate since the physiological rôle of the substance is still poorly understood. Whether a clinical entity caused solely by lack of vitamin P exists has not been established, and the relationship of vitamin P to vitamin C is still uncertain.

# Test 7

## THROMBOPLASTIN

### Preparation and Assay

*Definition.*—Thromboplastin* *is an essential coagulation factor* which reacts stoechiometrically with prothrombin, labile factor and calcium to form thrombin It is probably a lipo-protein which is widely distributed in body tissues, but in blood exists only in an inactive form, thromboplastinogen

*Principle.*—Attempts to isolate and purify thromboplastin have met with failure, since even mild chemical procedures tend to disrupt the molecule and reduce its activity Human and rabbit brain have an exceptionally high thromboplastic activity and serve, therefore, as an excellent source of this agent Rabbit brain is particularly suitable, since it is readily obtainable, and the tissue is almost bloodless after the removal of the pia and the larger vessels By dehydration with acetone, a product is obtained which has a high and remarkably constant activity When this material is kept in an evacuated vial, it remains fully active indefinitely

The simplest means for assaying thromboplastin is to test its action on fresh oxalated human or dog plasma using the one-stage technique, since the prothrombin in these two species is very constant By this method, the lowest concentration required to produce the shortest prothrombin time is determined An alternative method, which has not been fully developed, is to add varying quantities of the thromboplastin solution to be assayed to hemophilic plasma and after a period of incubation, determine by means of the prothrombin consumption test, how much prothrombin remains Since hemophilic blood is deficient in available thromboplastin, little prothrombin is consumed during coagulation, therefore, the amount of prothrombin which disappears becomes a measure of the quantity of thromboplastin added

*Equipment and Reagents*

Same as for prothrombin one-stage procedure, plus
Bone cutting forceps
Forceps

* The older term, thrombokinase, should be abandoned because the compound does not act as an enzyme

Scissors.
Suction flask (250 cc.).
Glass funnel (60 mm.).
Filter plate (25 mm.).
Filter paper.
Glass mortar (8 oz.) and pestle.
Acetone A good commercial grade is satisfactory, but a U S P. or C P product is recommended.

*Preparation of Thromboplastin from Rabbit Brain.*—It is advantageous to remove the brain soon after the rabbit is killed since the removal of blood vessels is then relatively easy. The technique of removing the brain is as follows: the skin over the head and neck is incised longitudinally in the midline and stripped back to expose the skull. By means of a bone forceps, the atlanto-occipital articulation is severed, and the plate of bone above the brain cut and removed The dura is then incised and the brain shelled out with the aid of a flat, blunt instrument such as the handle of a forceps. The pia is stripped and all visible vessels removed

To prepare enough thromboplastin reagent for approximately 500 prothrombin determinations, three cleaned rabbit brains weighing about 21 gm are put in an 8 oz glass mortar, covered with 30 cc of acetone, and 0 1 cc 0.2 M sodium citrate is added to remove any traces of calcium The material is crushed and mashed with the pestle, but grinding at this stage must be avoided The spent acetone is poured off, 25 cc. fresh acetone added and trituration repeated It will be noted that the second acetone extract is less milky and the material begins to become adhesive After the removal of the second portion of acetone, 25 cc fresh reagent are added At this stage the material becomes flaky and grinding may begin The acetone which is now almost water clear is poured off and a fourth quantity of 25 cc added The material is ground under the acetone until granular and non-adhesive The product is filtered by suction. A 25 mm filter plate fitted in an ordinary glass funnel and covered with a disc of filter paper is satisfactory for this purpose After washing repeatedly with small portions of fresh acetone, the material is dried first by suction, and then is transferred to a filter paper and dried at 37° C for 30 minutes The yield is about 3 5 gm of ivory white, finely granular material

If the product is used within 2 weeks, it is preserved as follows 0 2 gm quantities are distributed in test tubes (13 × 100 mm),

vacuum oil pump and sealed in a gas flame.* Precautions must be taken to have the material dry, since moisture will cause deterioration. Rapid evaporation of acetone during filtration is apt to cause condensation of moisture on the product especially if the atmosphere is warm and humid.

Fresh brains when frozen retain their full activity for 6 months It is, therefore, convenient to store the surplus supply of brains in the frozen state, and prepare only enough of the acetone dehydrated reagent for a two-week supply. In this manner, a hospital laboratory may provide itself with an adequate supply at a minimum amount of labor and expense.

*Assay of Thromboplastin.*—The amounts of reagent given in table 9 are put in test tubes mixed with 5 cc saline solution and incubated at 50° C for 20 minutes. The tubes are then placed in a water bath at 37° C Each solution is tested by the one-stage prothrombin procedure (Test 8) on fresh normal human plasma. Typical results are recorded in table 9 It will be noted that dog plasma, probably be-

TABLE 9—EFFECT OF CONCENTRATION OF THROMBOPLASTIN ON PROTHROMBIN TIME

| | *Prothrombin time in seconds* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Thromboplastin mg* * | *200* | *150* | *100* | *75* | *50* | *25* | *10* | *5* | *2 5* | *1* | *0* |
| Human plasma | | | | | | | | | | | |
| With 0 02 $CaCl_2$ | 13 | 13 | 13 | 13 | 14½ | 17 | 21 | 24 | 29 | 38 | 120 |
| With 0 01 $CaCl_2$ | 12 | 12 | 12 | 12 | 13 | 16 | 20 | 22 | 27 | 36 | 100 |
| Dog plasma | | | | | | | | | | | |
| With 0 02 $CaCl_2$ | 6 | 7 | 7½ | 8 | 9 | 11 | 12½ | 14 | 16 | 22 | 70 |
| With 0 01 $CaCl_2$ | 6 | 7½ | 8 | 9 | 10 | 12 | 13½ | 17 | 21 | 29 | 85 |

* The amount of thromboplastin mixed with 5 cc of saline solution and incubated at 50° C

cause of its greater concentration of prothrombin, requires more thromboplastin

*Application and Interpretation* —The most important use of thromboplastin is for the determination of prothrombin and for the prothrombin consumption test After preparing several hundred lots of this product by the method described, the writer can vouch for the constancy of its potency With this reagent a prothrombin time of 12 seconds for human and 6 seconds for dog plasma is consistently obtained Were this product accepted as the standard of reference, much of the unnecessary disagreement and confusion concerning prothrombin determination could be eliminated It should be stressed

* Samples kept in evacuated ampules have retained full activity for more than 12 years

that any thromboplastin preparation made of tissue from which blood is not easily removed and that applies especially to lung, is apt to contain clotting factors as contaminants. One can question the advisability of using such a product in research

Brain thromboplastin is free of labile factor, prothrombin and calcium To be sure, it contains much inert material but this does not have any detectable influence on the coagulation reactions

Rabbit brain thromboplastin is entirely satisfactory for the blood of man, dog, rabbit, cat and many other mammals It is not suitable for bird blood, nor should it be used for estimating the prothrombin of rat or guinea pig blood Thromboplastin from their brains is more active for their respective bloods

## QUANTITATIVE DETERMINATION OF THE CONSTITUENTS OF THE PROTHROMBIN COMPLEX

Much confusion exists at present concerning the factors that participate in the formation of thrombin For the sake of clarity, a rather arbitrary concept is presented Only four agents appear to be essential for the formation of thrombin They are: prothrombin, labile factor, organically bound calcium and thromboplastin. How these interact remains undetermined, but when their chemistry is studied in the natural environment, *i e*, in the blood or plasma, they behave as constituents in a stoechiometric reaction The quantity of prothrombin activated depends quantitatively on the amounts of labile factor, calcium and thromboplastin The report that prothrombin can spontaneously change to thrombin is of academic interest, but is of no help in furnishing, for example, an explanation for the coagulation defect in hemophilia If thromboplastin were acting as a catalyst, one would not expect the extremely low fixed consumption of prothrombin that one finds consistently in this disease

The recognition that prothrombin in human plasma exists partly in a free or reactive state, and partly in a precursor form, designated prothrombinogen, is of utmost importance. It offers a simple explanation for a number of experimental observations which have led some investigators to introduce hypothetical prothrombin-converting accelerators Prothrombinogen does not react until converted to prothrombin, but in contact with a foreign surface such as glass it gradually becomes activated, with the result that the free prothrombin is increased and the prothrombin time is shortened Unless this is recognized, the increased prothrombin activity may be erroneously ascribed to prothrombin accelerators All such hypothetical agents

should, therefore, be evaluated in relation to the activation of prothrombinogen

*Definitions.*—*Prothrombin* is the clotting factor which in its interaction with labile factor, calcium and thromboplastin yields thrombin. It is in the globulin fraction of the plasma, is inactivated at 58° to 60° C, is quantitatively adsorbed by tricalcium phosphate and completely eluted from the latter with sodium citrate. It is diminished in two types of congenital hypoprothrombinemias described by Quick, in vitamin K deficiency, after the administration of Dicumarol and in some types of liver damage It reacts with sodium citrate, apparently forming a complex, which is unadsorbable by tricalcium phosphate, and is inactive

*Prothrombinogen* is a precursor of prothrombin In human blood its concentration is several times greater than that of active prothrombin In contact with a foreign surface such as glass, prothrombinogen is slowly converted to the active form Neither its rôle nor how it is activated physiologically is known It is inactivated at 60° C, is adsorbed by tricalcium phosphate and eluted with sodium citrate.

*The labile factor* is an essential agent in the formation of thrombin It diminishes in stored plasma due presumably to destruction by oxidation Its instability is greatly increased by the absence of calcium ions Like prothrombin, it is inactivated at 60° C but unlike this agent it is not adsorbed by tricalcium phosphate nor is it diminished in vitamin K deficiency or after the administration of Dicumarol It is decreased in severe liver damage and in the type of congenital hypoprothrombinemia described by Owren Its concentration is relatively low in human blood—dog blood contains ten times and rabbit blood fifty times more Other names applied to this substance are Factor V, Ac-globulin, acceleration factor, thromboplastin co-factor

*Calcium* —The rôle of this element in coagulation remains unknown Only organically bound calcium appears to react, and it seems reasonable to postulate that one of the prothrombin factors is a calcium compound The minimum calcium concentration for maximum prothrombin activity is 0 0012 M for human and 0 0004 M for dog plasma Curiously, when the prothrombin level decreases, the calcium concentration required for maximum activity increases

## Test 8

### DETERMINATION OF PROTHROMBIN

One-Stage Procedure

*Definition* – The determination of prothrombin by means of adding thromboplastin reagent and calcium chloride to oxalated plasma, and timing the formation of a clot is commonly designated as the one-stage method

*Principle* – The clotting of blood is slow, because the available thromboplastin is limited in amount. On adding an excess of the latter, prothrombin is made the limiting factor and therefore the determinant of the clotting time The basic over-all equation is

Prothrombin + labile factor + calcium + thromboplastin = thrombin

The reaction behaves stoechiometrically, therefore, since labile factor and calcium are above the minimum required concentration and thromboplastin is made a constant by adding an excess, the amount of thrombin formed depends on prothrombin which becomes the variable in the equation Since the concentration of thrombin is determined by the speed of coagulation, the clotting time becomes the measure of the prothrombin level of the blood For convenience, the

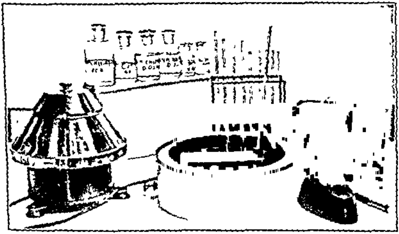


FIG 11 – The equipment for the determination of prothrombin and prothrombin consumption.

test is carried out on oxalated rather than on native plasma A fixed amount of sodium oxalate is added to a specified volume of blood, and a standardized quantity of calcium chloride is added to the plasma in the test The prothrombin concentration is determined from the clotting or "prothrombin" time using the table of values which the writer has prepared with great care from data obtained by various types of study and from normal as well as pathological bloods.

*Equipment and Reagents.*

Water bath

The Technicon water bath is quite satisfactory An inexpensive one can be made using a round glass dish 20 inches in diameter and 4 inches deep and placing it over a shallow box containing two 40-watt light bulbs which serve as the source of heat.

Copper test tube rack

Thermometer

Centrifuge

Vacuum or thermos bottle

Stop watch

Test tubes, 13 × 100 mm.

Pipettes—1 cc serological graduated in tenth's and hundredth's of a cc For convenience, the pipette should be shortened to 170 mm

Sodium oxalate 0 1 M

Calcium chloride 0 02 M

Calcium chloride 0 01 M

*Thromboplastin Solution* —0 2 gm acetone dehydrated rabbit brain (*See* Test 7) is put in a test tube, covered with 5 cc saline solution and mixed by blowing through the suspension with a pipette The powder must not be triturated in a mortar with saline since this causes a loss of activity The solution is incubated at 50° C for 20 minutes A vacuum bottle with a cork into which the test tube can be inserted serves as a suitable incubating bath The tube is transferred to the water bath kept at 37° C and the contents occasionally agitated by blowing through it since this aids in maintaining its activity The suspension settles rapidly and the solid particles remaining do not interfere with the accuracy of the test, in fact they actually aid in the detection of the incipient clot

*Procedure* —Nine volumes of blood obtained by venipuncture with a silicone coated syringe and needle are mixed with one volume of 0 1 M sodium oxalate Usually 2 cc of blood are adequate As small as 0 36 cc of blood mixed with 0 04 cc sodium oxalate is sufficient for 2 prothrombin determinations If blood cannot be obtained by venipuncture, capillary blood may be used A deep skin cut is

made and the blood allowed to drop into a conical centrifuge tube graduated for 1 cc, and containing 0.1 cc of 0.1 M. sodium oxalate. It is convenient to remove plasma from the centrifuged blood by means of a pipette having a rubber bulb from a medicine dropper attached to the end

The prothrombin determination is carried out as follows: 0.1 cc. of plasma is transferred to a standard test tube and 0.1 cc. of thromboplastin added The tube is placed in a water bath for a few seconds Exactly 0.1 cc. of 0.01 M. calcium chloride is measured into a pipette which is put in the mouth, so that the reagent can be blown forcibly into the tube, thus assuring almost instantaneous mixing with the plasma and thromboplastin The stop watch is clicked at the moment the calcium chloride is added. The tube is placed in the water bath and shaken lightly. A few seconds before the expected clotting time, the tube is removed from the bath and held toward a distant source of light, and the tube observed from below By very gently tilting the tube, the incipient web of fibrin, which is the end point, can readily be detected If the tube is shaken too vigorously, the initial fibrin clot may be broken, and so escape detection More time will, therefore, be required before sufficient fibrin will again be formed to become visible, and this may introduce an appreciable error.

The determination is repeated using 0.02 M calcium chloride, which is the concentration formerly recommended With the introduction of silicone-coated glassware, the conversion of a small amount of prothrombinogen is prevented, and the mild inhibitory action of calcium is accentuated by 0.02 M calcium chloride. Thus, normal oxalated human plasma with 0.02 M calcium chloride yields a prothrombin time of 13 seconds whereas with 0.01 M calcium chloride, it is 12 seconds The second value is the correct one, because native plasma when mixed with thromboplastin has a prothrombin time of 12 seconds When the prothrombin level drops, the stronger solution of calcium must be used, since the calcium requirement increases as the prothrombin decreases For dog blood, 0.02 M. calcium chloride is satisfactory In any case, the concentration of calcium chloride is selected which yields the shortest prothrombin time

In research studies when great accuracy is desired, the prothrombin should be determined within 30 minutes after the blood is drawn Four hours storage is permissible for routine clinical purposes It should be remembered that two changes occur in oxalated human plasma, the labile factor decreases and prothrombinogen is activated to prothrombin If the test tube containing the blood is kept in cold water, both reactions proceed very slowly

When the prothrombin time is very greatly prolonged, it is conven-

ient to use as a miniature water bath, a large test tube (25 × 100 mm.) fitted with a cork having a hole through which the smaller test tube (13 × 100 mm.) can be inserted and held in place. The larger tube is filled with water at 37½° C This device allows continuous observation while the temperature of the reacting medium is maintained constant. When the prothrombin time is plotted against the prothrombin concentration of plasma, a hyperbolic curve is obtained which is fairly satisfactorily expressed by the equation:

$$\text{Prothrombin (Per cent of normal)} = \frac{k}{\text{p t.} - a}$$

(p.t = prothrombin time, k and a are constants For human plasma the values for k and a are 303 and 8 7 respectively )

In table 10 the values of prothrombin times in concentration of normal for human and dog plasma are presented.

It should be emphasized that these values are valid only when the procedure is carried out exactly as outlined and when a fully potent thromboplastin is used

*Application and Interpretation* —It is generally agreed that the one-stage procedure for determining prothrombin is satisfactory in meeting clinical needs The writer, as the result of seventeen years of intensive study of prothrombin, can further vouch that the method fulfills the precise requirements of research and is the most reliable quantitative method for prothrombin available today A test which easily detects the difference in prothrombin response between 125 and 100 gammas of vitamin K in a 26-pound dog (*See* Table 7, page 76) compares favorably with other biochemical procedures for estimating the constituents of the blood, especially the clotting factors

The first wide application of the method was in determining quantitatively the decrease of prothrombin in vitamin K deficiency, and in testing the efficacy of vitamin K By means of the test, it was found that the prothrombin of the newborn often drops to very low levels during the first few days of life and usually returns to normal in a week The hypoprothrombinemia in obstructive jaundice was studied with the test even before vitamin K was known In both types of avitaminosis K, the test is entirely reliable, since only prothrombin becomes deficient In sprue, a lack of vitamin K may occur, which causes a decrease of prothrombin, likewise sterilization of the intestinal tract by means of antibiotics or sulfonamide drugs may decrease the supply of vitamin K below the daily requirement The

fall in prothrombin is accurately measured by the one-stage procedure.

Liver damage causes a decrease of prothrombin and occasionally also a reduction of the labile factor Since the concentration of the labile factor even in normal human blood is relatively low, a moderate reduction will prolong the prothrombin time. The test will, therefore, no longer be a true measure of prothrombin. By mixing 3 parts of deprothrombinized rabbit plasma with 7 parts of the plasma to be tested, the labile factor is restored and a fairly accurate estimate of prothrombin from the prothrombin time of the mixture can be made.

The one-stage method has served as a reliable and accurate test for estimating prothrombin in Dicumarol therapy. By means of this test it has recently been found that in man Dicumarol first causes a drop of free prothrombin,[1] and a decrease of the prothrombinogen only after the drug is given several days The labile factor level is not altered by Dicumarol.

The test has made it possible to diagnose and differentiate three types of congenital hypoprothrombinemias. In the first type, the labile factor is diminished In applying the one-stage procedure, it is necessary to supply this factor This is readily accomplished by mixing 3 parts of deprothrombinized rabbit plasma with 7 parts of the plasma to be tested The mixture has a prothrombin time which corresponds to the true prothrombin content In both of the other two types, the labile factor is normal, but the free prothrombin is diminished In one, however, the prothrombinogen concentration is normal whereas in the other it is diminished

From this survey, it is clear that the one-stage procedure without any modification determines accurately all the common types of hypoprothrombinemias Only in the exceedingly rare condition of congenital deficiency of labile factor and in an occasional case of liver damage does the procedure require the slight modification of adding labile factor in the form of deprothrombinized rabbit plasma To be sure the prothrombin time is not a true measure of prothrombin when heparin is added but physiological heparinemia is practically unknown It is obvious that in afibrinogenemia, the prothrombin time cannot be determined unless fibrinogen is added Considering that no case is known in the United States outside of Boston, this becomes merely of academic interest

In spite of the wide and successful employment of the one-stage procedure in the clinical laboratory, the method has been repeatedly attacked as not being an accurate quantitative test for prothrombin Some writers have gone as far as to recommend that the name be changed to "accelerated clotting time" This hostile attitude toward

the method stems from the fact that soon after the one- and two-stage methods were described, a number of workers postulated that there was a prothrombin conversion factor, which determined the speed of thrombin formation. The one-stage method, according to this hypothesis, measures the conversion factor rather than prothrombin The two-stage method, on the other hand, because it eliminates this factor by allowing all the prothrombin to become activated before measuring thrombin, was proclaimed as the standard method for determining prothrombin. Therefore, whenever the one-stage method did not agree with the two-stage test, it was condemned as unsatisfactory So far the hypothetical conversion factor has not been isolated It was thought for a while that the labile factor was the sought-for conversion agent, but recent studies show that it, like thromboplastin, reacts stoechiometrically with prothrombin. With the discovery that prothrombin may exist in an inactive form, the *differences between the one- and two-stage methods can readily* be accounted for without postulating a conversion factor. The one-stage procedure measure free prothrombin only, whereas the two-stage determines the free, plus whatever amount of prothrombinogen becomes converted to prothrombin during the various manipulations Since it is only the free prothrombin which appears to react in the hemostatic response, it becomes more important than ever that the one-stage method be regarded and treated as a precise tool of research. There is no longer any justification for papers to appear in scientific journals in which for instance the normal prothrombin time is recorded as 15 to 20 seconds for human plasma If the normal prothrombin time cannot be determined within one-half second, it is advisable that the investigator should first learn how to do the test correctly before attempting research

The one-stage procedure as outlined is the product of many years of meticulous and painstaking studies In the seventeen years since its inception, only one modification was introduced, namely substituting acetone dehydrated rabbit brain, for fresh or air-dried brain Recently, when the routine use of silicone-coated glassware was begun, it was found that a 0 01 M calcium chloride solution gave a more accurate prothrombin time value for normal human plasma than the 0 02 M solution Since the stronger concentration of calcium is needed for dog plasma, and when the prothrombin is low, the prothrombin time is best determined with both solutions of calcium chloride.

Various modifications of the one-stage method have been published None has improved the determination either in accuracy or in simplification. Some have definitely reduced the accuracy of the test. A

short analytical review of the common modifications should be helpful

Sodium oxalate was chosen as the anticoagulant, but equivalent amounts of potassium or ammonium oxalate are equally satisfactory. Likewise, 0 1 or 0 2 M. sodium citrate can be used in place of 0.1 M.

the test if the prothrombin determination is done within 2 hours after the blood is collected.

Dilution of plasma has been proposed as a means of increasing the sensitivity of the test. Since the prothrombin time in human blood increases only 3 seconds when the prothrombin decreases from 100 to 50 per cent, dilution with an equal volume of physiological saline is helpful to obtain a slightly greater accuracy in that range Since the prothrombin time should and can be determined with an accuracy within one-half second, higher dilutions or dilution of plasmas having a prothrombin time greater than 15 seconds are unnecessary. The $12\frac{1}{2}$ *per cent diluted plasma modification must be condemned, since* at such a high dilution uncontrolled physio-chemical changes are introduced, and furthermore, accurate timing of the clot becomes difficult because of the low concentration of fibrinogen

The use of deprothrombinized plasma (*i e* , barium sulfate treated) as a diluting agent has introduced a grave error into the determination of prothrombin in human blood Barium sulfate like tricalcium phosphate removes prothrombin by adsorption, but it does not take out a factor which converts prothrombinogen to free prothrombin Therefore, when plasma treated with barium sulfate is added to normal plasma, some of the prothrombinogen of the latter is activated This is made clear by the data in table 11 It will be observed that the prothrombin time remains constant until the quantity of barium sulfate plasma exceeds 50 per cent This type of plasma, therefore, is unsatisfactory for constructing a prothrombin curve Deprothrombinized plasma is a valuable agent, but it must be recognized that it is more than a solution of fibrinogen It contains nearly all the labile factor, as well as the agent that apparently keeps the balance between active prothrombin and prothrombinogen

The substitution of *acetone dehydrated rabbit brain thromboplastin* with other preparations has led to unsatisfactory, and in one instance, to disastrous results It has been found that Russell viper venom fails utterly in the determination of prothrombin in human plasma following the administration of Dicumarol The recent literature on the subject has been reviewed by the author [2]

Thromboplastin obtained from a tissue such as lung, which is difficult to free from blood, is apt to contain the factor which converts prothrombinogen to prothrombin. Such a product cannot be recommended for research unless its purity is established.

TABLE 10.—THE VALUES OF PROTHROMBIN TIMES IN TERMS OF PER CENT OF NORMAL

| *Prothrombin Time* | *Per Cent of Normal* | |
|---|---|---|
| *Sec* | *Dog* | *Man* |
| 6 | 100 | |
| 7 | 50 | |
| 8 | 40 | |
| 9 | 25 | |
| 10 | 20 | |
| 12 | 10 | 100 |
| 13 | 7 | 7 |
| 14 | 6 | 60 |
| 15 | 5 | 50 |
| 17 | 4 | 40 |
| 18 | 3.5 | 35 |
| 19½ | 3 | 30 |
| 21–22 | 2.5 | 25 |
| 24–26 | 2.0 | 20 |
| 37–40 | 1 | 10 |
| 55–65 | 0.5 | 5 |

Thromboplastin from human brain is very active, often even more than rabbit brain. Thus far it has not been standardized as carefully as has rabbit brain. It is not permissible to apply prothrombin values obtained with human brain to those of rabbit brain.

While a difficult procedure in surgery is rarely a deterrent to its widespread use, the clamor for simplification of laboratory tests is incessant. It is no wonder, therefore, that bedside tests have a special appeal. In 1935 Quick and his associates[3] used such a test to show that the prothrombin was normal in hemophilia. The test consisted in adding 0.1 cc. of thromboplastin to 1 cc. of freshly drawn blood. Four years later Ziffren *et al*[4] proposed a similar test. They added 0.9 cc. of blood to 0.1 cc. of thromboplastin. Unfortunately they proposed a prothrombin index calculated by dividing the clotting time of the normal control by the clotting time of the patient's blood. Since the relation of concentration of prothrombin to clotting time is not linear, but is expressed by a hyperbolic curve, a serious error is introduced when the prothrombin index is confused with the per cent of prothrombin concentration. The bedside test also has other sources of error. The delivery of 0.9 cc. of blood and mixing it with thromboplastin requires several seconds. This makes accurate

TABLE II.—THE EFFECT OF DILUTING HUMAN PLASMA WITH DEPROTHROMBINIZED PLASMA (PREPARED BY ADSORPTION WITH $Ca_3(PO_4)_2$ AND $BaSO_4$) ON THE PROTHROMBIN TIME

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal oxalated human plasma | cc | 0.1 | 0.09 | 0.08 | 0.7 | 0.06 | 0.05 | 0.04 | 0.03 | 0.02 | 0.01 | 0 |
| Human plasma treated with $Ca_3(PO_4)_2$ | cc | 0 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 | 0.1 |
| Prothrombin time | sec | 12 | 12 | 12 | 12 | 12 | 12 | 13 | 14 | 17 | 21 | ∞ |
| | | | | | | | | | | | | |
| Normal oxalated human plasma | cc | 0.1 | 0.09 | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 | 0.02 | 0.01 | 0 |
| Human plasma treated with $BaSO_4$ | cc | 0 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 | 0.1 |
| Prothrombin time | sec | 12 | 12 | 12 | 12 | 12 | 12 | 12½ | 13½ | 16 | 19 | ∞ |
| | | | | | | | | | | | | |
| Normal oxalated human plasma | cc | 0.1 | 0.09 | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 | 0.02 | 0.01 | 0 |
| Saline solution | cc | 0 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 | 0.1 |
| Prothrombin time | sec | 12 | 12 | 12½ | 13 | 14 | 15 | 17 | 19 | 24 | 38 | ∞ |

Thromboplastin obtained from a tissue such as lung, which is difficult to free from blood, is apt to contain the factor which converts prothrombinogen to prothrombin. Such a product cannot be recommended for research unless its purity is established

TABLE 10 – THE VALUES OF PROTHROMBIN TIMES IN TERMS OF PER CENT OF NORMAL

| *Prothrombin Time Sec* | *Per Cent of Normal* Dog | Man |
|---|---|---|
| 6 | 100 | |
| 7 | 50 | |
| 8 | 40 | |
| 9 | 25 | |
| 10 | 20 | |
| 12 | 10 | 100 |
| 13 | 7 | 7 |
| 14 | 6 | 60 |
| 15 | 5 | 50 |
| 17 | 4 | 40 |
| 18 | 3.5 | 35 |
| 19½ | 3 | 30 |
| 21–22 | 2.5 | 25 |
| 24–26 | 2.0 | 20 |
| 37–40 | 1 | 10 |
| 55–65 | 0.5 | 5 |

Thromboplastin from human brain is very active, often even more than rabbit brain. Thus far it has not been standardized as carefully as has rabbit brain. It is not permissible to apply prothrombin values obtained with human brain to those of rabbit brain

While a difficult procedure in surgery is rarely a deterrent to its widespread use, the clamor for simplification of laboratory tests is incessant It is no wonder, therefore, that bedside tests have a special appeal In 1935 Quick and his associates[4] used such a test to show that the prothrombin was normal in hemophilia The test consisted in adding 0.1 cc of thromboplastin to 1 cc of freshly drawn blood Four years later Ziffren *et al*[5] proposed a similar test. They added 0.9 cc of blood to 0.1 cc of thromboplastin Unfortunately they proposed a prothrombin index calculated by dividing the clotting time of the normal control by the clotting time of the patient's blood Since the relation of concentration of prothrombin to clotting time is not linear, but is expressed by a hyperbolic curve, a serious error is introduced when the prothrombin index is confused with the per cent of prothrombin concentration The bedside test also has other sources of error The delivery of 0.9 cc of blood and mixing it with thromboplastin requires several seconds This makes accurate

## Test 9

# PROTHROMBIN DETERMINATION BY THE ADSORPTION AND ELUTION TECHNIQUE

*Definition* —The estimation of prothrombin by adsorbing it with tricalcium phosphate, eluting it by means of sodium citrate, and assaying the eluate by adding it to standard deprothrombinized plasma and determining the prothrombin time of the reconstituted plasma can be designated as the adsorption and elution method.

*Principle.*—Tricalcium phosphate has a high specificity for adsorbing prothrombin A small amount is sufficient to remove quantitatively the prothrombin from oxalated or native plasma Equally effective and specific is sodium citrate in eluting prothrombin from the adsorbent By mixing the eluate with deprothrombinized plasma, a reconstituted plasma is obtained on which the prothrombin time can be determined Rabbit plasma adsorbed with tricalcium phosphate is ideal as the testing medium since it has a high concentration of labile factor and a high constancy of composition The scheme of the determination is presented in figure 9

*Equipment and Reagents*·

Same as the one-stage prothrombin test, plus.

Pipettes calibrated in tenths and hundredths of a cc

Sodium citrate—0 2 M

Tricalcium phosphate suspension 0 005 M

It is prepared by mixing 2 5 cc of stock 0 2 M tricalcium phosphate with 97 5 cc of distilled water The tricalcium phosphate in 1 cc of this suspension is sufficient to adsorb the prothrombin from 1 cc. of human plasma. For rabbit and dog plasma 2 5 cc of 0 005 M $Ca_3(PO_4)_2$ are needed to remove the prothrombin from 1 cc

Preparation of stock 0 2 M tricalcium phosphate 158 gm of trisodium phosphate C P are dissolved in 1000 cc of distilled water In a separate container 66 6 gm of anhydrous calcium chloride C P are dissolved in 1000 cc of distilled water The trisodium phosphate solution is thoroughly stirred while the calcium chloride is slowly added When the reaction is completed, the pH is adjusted to 7 with dilute hydrochloric acid The precipitated tricalcium phosphate is transferred to a 4-liter jar and washed repeatedly by decantation At first tap water may be used, but the final washing must be with dis-

timing difficult. The exact moment of clotting is not easy to determine in whole blood. Another disadvantage is that unless a thromboplastin of constant potency is employed, each test requires a normal control. The most serious criticism of the test, however, is that it is the type of procedure that invites carelessness and indifference.

Since many of the various modifications have definitely impaired the accuracy and reliability of the one-stage method, it seems reasonable to request that no one should attempt modifying the test until he has mastered the original technique. It is quite probable that in this way much talent and effort can be conserved for more productive achievements. There is little justification for semi-quantitative methods such as the various types of bedside procedures. With a little more effort a precise quantitative determination can be made. When such potent drugs as Dicumarol are administered, accurate estimation of prothrombin for the sake of the patient's safety is mandatory.

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substantiate the correctness and accuracy of the one-stage method. A remarkable agreement is found between the results obtained by the two methods Only when the labile factor is lacking in plasma do the results differ.

This method should end the speculation of a prothrombin conversion accelerator factor since it is unlikely that if such an agent should exist, it would, like prothrombin, be quantitatively adsorbed and eluted. The calcium phosphate treated rabbit plasma which serves as the standard testing medium has a high concentration of labile factor, but in spite of this excess, the reaction is not accelerated.

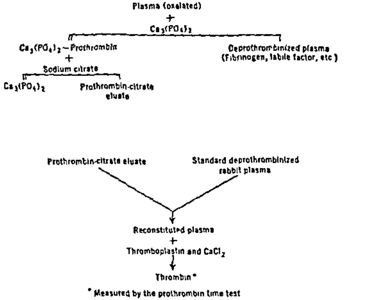



FIG. 12 – The adsorption and elution of prothrombin as a means of assay

*Proposed Unit of Prothrombin* The amount of prothrombin either as an eluate or in purified form, which, when added to 1 cc rabbit plasma deprothrombinized with tricalcium phosphate will yield a prothrombin time of 12 seconds is 1 unit. According to this proposal 1 cc of fresh human blood normally contains 1 unit of free or active prothrombin

## BIBLIOGRAPHY

1 QUICK, A J and STEFANINI, M The concentration of component A in blood, its assay and relation to the labile factor, J Lab and Clin Med, 34, 973, 1949

tilled water. When the sodium chloride is removed, the volume of the tricalcium phosphate reagent is brought to 1 liter, thus making a 0.2 M suspension. It requires several weeks to prepare the reagent.

Preparation of deprothrombinized rabbit plasma: Transfer 12.5 cc. of 0.005 M. tricalcium phosphate to a test tube and centrifuge at 2500 r.p.m. for 3 to 5 minutes to pack the adsorbent. The clear water is poured off and the tube drained. Five cc. of fresh oxalated rabbit plasma are mixed with the tricalcium phosphate by means of a stirring rod. After 5 minutes, the tube is centrifuged at 2500 to 3000 r.p.m. for 10 minutes. The clear plasma is poured from the packed adsorbent into a clean test tube and kept in an ice bath. No clot should form when 0.1 cc. of this plasma is mixed with 0.1 cc. of thromboplastin and 0.1 cc. of 0.02 M. calcium chloride.

*Procedure*[1]—The determination for human plasma is as follows: 2 cc. of 0.005 M. calcium phosphate suspension are transferred to a test tube and centrifuged for 3 to 5 minutes to pack the adsorbent. The water is poured off and the tube drained. To the packed tricalcium phosphate, 2 cc. of the oxalated plasma to be analyzed is added. The adsorbent and plasma are mixed with a stirring rod for 2 minutes and allowed to stand 3 more minutes. The tube is centrifuged at a minimum speed of 2500 r.p.m. for 5 minutes. The clear plasma is poured off and the tube drained for several minutes. The adsorbent is washed by adding 5 cc. saline solution and stirring with a glass rod. The adsorbent is again packed by centrifugation as before. The wash water is poured off and the tube inverted to drain. The water on the side of the tube is carefully removed by means of filter paper. Exactly 0.2 cc. of 0.2 M. sodium citrate is added to the packed adsorbent. The mixture is stirred for 3 minutes and allowed to stand 2 minutes more before it is centrifuged for 5 minutes, at 2500 r.p.m. The 0.2 cc. of sodium citrate eluate contains all the prothrombin originally present in 2 cc. of plasma. The prothrombin has, therefore, been concentrated ten fold. By diluting 1 volume of the eluate with 9 volumes of deprothrombinized rabbit plasma, the reconstituted plasma contains the same amount of prothrombin as the original plasma. Therefore, 0.01 cc. measured with a 0.1 cc. pipette is transferred to a test tube and 0.09 cc. of deprothrombinized rabbit plasma added. To this mixture 0.1 cc. of thromboplastin and 0.1 cc. of 0.02 M. calcium chloride are added in the same manner as in the one-stage procedure and the clotting time carefully determined. The prothrombin can be estimated from the clotting time using the prothrombin curve or table.

*Application and Interpretation.*—The new method of determining prothrombin by adsorption and elution has thus far served mainly to

substantiate the correctness and accuracy of the one-stage method. A remarkable agreement is found between the results obtained by the two methods. Only when the labile factor is lacking in plasma do the results differ.

This method should end the speculation of a prothrombin conversion accelerator factor since it is unlikely that if such an agent should exist, it would, like prothrombin, be quantitatively adsorbed and eluted. The calcium phosphate treated rabbit plasma which serves as the standard testing medium has a high concentration of labile factor, but in spite of this excess, the reaction is not accelerated.

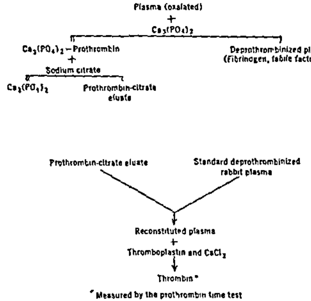



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Preparation of deprothrombinized rabbit plasma: Transfer 12 5 cc of 0 005 M. tricalcium phosphate to a test tube and centrifuge at 2500 r p m for 3 to 5 minutes to pack the adsorbent. The clear water is poured off and the tube drained. Five cc. of fresh oxalated rabbit plasma are mixed with the tricalcium phosphate by means of a stirring rod. After 5 minutes, the tube is centrifuged at 2500 to 3000 r p m. for 10 minutes. The clear plasma is poured from the packed adsorbent into a clean test tube and kept in an ice bath. No clot should form when 0.1 cc. of this plasma is mixed with 0 1 cc. of thromboplastin and 0 1 cc. of 0 02 M calcium chloride.

*Procedure* [1] —The determination for human plasma is as follows· 2 cc of 0 005 M calcium phosphate suspension are transferred to a test tube and centrifuged for 3 to 5 minutes to pack the adsorbent. The water is poured off and the tube drained To the packed tricalcium phosphate, 2 cc of the oxalated plasma to be analyzed is added. The adsorbent and plasma are mixed with a stirring rod for 2 minutes and allowed to stand 3 more minutes The tube is centrifuged at a minimum speed of 2500 r p m for 5 minutes The clear plasma is poured off and the tube drained for several minutes. The adsorbent is washed by adding 5 cc saline solution and stirring with a glass rod The adsorbent is again packed by centrifugation as before The wash water is poured off and the tube inverted to drain. The water on the side of the tube is carefully removed by means of filter paper Exactly 0 2 cc of 0 2 M sodium citrate is added to the packed adsorbent The mixture is stirred for 3 minutes and allowed to stand 2 minutes more before it is centrifuged for 5 minutes, at 2500 r p m The 0 2 cc of sodium citrate eluate contains all the prothrombin originally present in 2 cc of plasma The prothrombin has, therefore, been concentrated ten fold By diluting 1 volume of the eluate with 9 volumes of deprothrombinized rabbit plasma, the reconstituted plasma contains the same amount of prothrombin as the original plasma Therefore, 0 01 cc measured with a 0 1 cc pipette is transferred to a test tube and 0 09 cc of deprothrombinized rabbit plasma added To this mixture 0 1 cc of thromboplastin and 0 1 cc of 0 02 M. calcium chloride are added in the same manner as in the one-stage procedure and the clotting time carefully determined The prothrombin can be estimated from the clotting time using the prothrombin curve or table

*Application and Interpretation* —The new method of determining prothrombin by adsorption and elution has thus far served mainly to

sodium oxalate. The plasma obtained by centrifugation is treated with tricalcium phosphate to remove prothrombin according to the procedure of Test 9.

As a preliminary step, 0 01 cc. of the deprothrombinized plasma is added to 0 09 cc. of the standard stored human plasma The prothrombin time of this mixture is accurately determined by means of the one-stage procedure If a value less than 20 seconds is obtained, the [illegible]

12 serves as a guide Thus, in determining the concentration of labile

TABLE 12 — THE CONCENTRATION OF LABILE FACTOR IN THE PLASMA OF RABBIT, DOG AND MAN AS TESTED BY ITS EFFECT TO REDUCE THE PROTHROMBIN TIME OF STORED HUMAN PLASMA

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Volume of stored human plasma | | 100 | 99 9 | 99 8 | 99 5 | 99 | 98 | 95 | 90 | 80 | 70 |
| Volume of plasma to be assayed | | | 0 1 | 0 2 | 0 5 | 1 | 2 | 5 | 10 | 20 | 30 |
| | | *Prothrombin time in seconds* | | | | | | | | | |
| Type of plasma | rabbit | 70 | 25 | 20 | 16 | 15 | 11 | 9 | 8 | 8 | 7 |
| | dog | | | | | 20 | 16 | 13 | 11 | 8 | 7½ |
| | man | | | | | | | | 20 | 16 | 14 |

factor in fresh rabbit plasma, it will be found that one part of plasma must be mixed with 49 parts of saline to give a mixture which, if 0 01 cc of it is mixed with 0 09 cc of stored human plasma, will produce a prothrombin time of 20 seconds Since rabbit plasma requires a 1 to 50 dilution, its concentration of labile factor is 50 times that of normal human plasma or 1 cc of rabbit plasma contains 500 units

If a plasma does not shorten the prothrombin time to 20 seconds or less when mixed with 9 volumes of stored plasma, it indicates that the concentration of the labile factor is less than 10 units per cc , or in other words, it is below the level of normal human plasma Since it is difficult to obtain satisfactory analytical results in this low range, it is best simply to determine whether adding deprothrombinized rabbit plasma will reduce the prothrombin time to normal If it does not, one can conclude that the prothrombin is also diminished It is suggested, therefore, to determine the prothrombin with the adsorption-elution technique, (Test 9) The following experimental data from Dr Collentine's studies serve to illustrate the approach·

Dog B —The animal was fasted 24 hours, then given chloroform anesthesia for 1½ hours The blood was collected 48 hours later The results were

Prothrombin time 59 sec

## Test 10

### THE DETERMINATION OF THE LABILE FACTOR

*Principle* —When oxalated human plasma is stored at refrigerator temperature (4 to 5° C ) the prothrombin time gradually increases due to the progressive loss of labile factor By re-adding labile factor to the stored plasma, the prothrombin time is shortened in proportion to the amount added The stored plasma may, therefore, be made to serve as an easily obtainable assay medium. The restoration of the prothrombin time can be standardized so that it becomes a quantitative measure of the labile factor. In assaying plasma or serum it is necessary first to remove prothrombin This is readily accomplished by adsorption with tricalcium phosphate which at the most removes only a trace of the labile factor This adsorbent does not remove prothrombin from citrated plasma, therefore, if an anticoagulant is used, it should be sodium oxalate

*Unit of Labile Factor* —When 0 1 cc of deprothrombinized fresh human oxalated plasma is added to 0.9 cc. of stored human plasma with a prothrombin time of 40 to 60 seconds, the mixture will have a prothrombin time of 20 to 21 seconds This serves to establish the unit, which may be defined as the amount of labile factor that when added to 0 9 cc of stored human plasma will reduce the prothrombin time to 20 seconds According to this definition, 0 1 cc. of fresh human plasma contains 1 unit of labile factor or 1 cc has a concentration of 10 units

*Apparatus and Reagents*

Same as in Tests 8 and 9, plus

Stored oxalated human plasma. Blood is obtained by venipuncture and mixed with 0 1 M sodium oxalate in the proportion of 9 to 1 After centrifugation, the plasma is separated and transferred to small test tubes (13 × 100 mm ) The tubes are placed in a refrigerator unstoppered. It is best to use silicone glassware in collecting this blood, but storage must be in glass When the prothrombin time has dropped to about 40 seconds, which requires approximately two weeks, the tubes of plasma are frozen and kept in the freezing compartment of the refrigerator

*Procedure.*[1] —The blood to be tested is collected in the usual manner by venipuncture and mixing 9 volumes with 1 volume of 0 1 M.

it is stored The labile factor diminishes, presumably because it is oxidized, and the more the calcium ions are depressed, the greater the rate of destruction. This explains why the prothrombin time increases much more rapidly when 0.1 M sodium oxalate is used than when 0.1 M. sodium citrate is the anticoagulant. The second important reaction during storage is the conversion of prothrombinogen to prothrombin This occurs faster when 0 1 M. sodium citrate is used instead of sodium oxalate. This accounts for the observation, which once was very puzzling, that human plasma mixed with 0 1 M. sodium citrate will after 24 hours have a prothrombin time of 9 to 10 seconds

Stored human plasma is equally as satisfactory as fresh plasma for correcting true hypoprothrombinemia by transfusion. It is worthless in the type of case reported by Owren and it is of questionable value when severe liver damage is a factor in the hypoprothrombinemia since it is likely that the labile factor is also diminished

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| | |
|---|---|
| Stored human plasma | 0 09 cc |
| Dog B Plasma $Ca_3(PO_4)_2$ treated | 0 01 cc. |
| Prothrombin time | 26 sec |
| | |
| Dog B plasma | 0 09 cc |
| Eluate from plasma of normal dog | 0 01 cc |
| Prothrombin time | 32 sec |
| | |
| Rabbit plasma $Ca_3(PO_4)_2$ treated | 0 09 cc |
| Eluate from Dog B plasma | 0 01 cc |
| Prothrombin time | 29 sec |
| | |
| Rabbit plasma $Ca_3(PO_4)_2$ treated | 0 09 cc |
| Eluate from plasma of normal dog | 0 01 cc |
| Prothrombin time | 6 sec |

These results show clearly that the prolonged prothrombin time observed in the dog given chloroform anesthesia is the result of a deficiency of both prothrombin and of labile factor. The results further indicate that the true prothrombin time, *i.e*, the value that would be obtained were there no lack of labile factor, would be approximately 29 seconds

*Application and Interpretation.*—The quantitative determination of the labile factor in blood is rarely required in clinical studies. Even in human blood though the level is relatively low, the amount is still in excess of that required for the maximum reaction with prothrombin In the common types of hypoprothrombinemia such as induced by vitamin K deficiency and by Dicumarol, the labile factor is not affected

Until now a reduction of labile factor in vivo has only been found as a congenital anomaly and in severe liver damage Congenital lack of labile factor is exceedingly rare, and no case has yet been reported in North America In chronic liver disease, a moderate drop in labile factor may occasionally be observed Dr Collentine has recently found, as shown in the above results, a drastic reduction in acute liver damage induced by chloroform poisoning accompanied by an equally drastic drop in prothrombin This suggests that, perhaps, a severe depression of labile factor occurs in acute yellow atrophy of the liver

When the concentration of the labile factor is low, a bleeding tendency occurs even though the prothrombin is normal This is well illustrated by Owren's case [2] In animals such as the dog, rabbit and cow, it is improbable that a deficiency of this agent is ever severe enough to produce hemorrhage, because of the large excess which is normally present

The relation of labile factor to storage of plasma is of practical significance Two important changes occur in human plasma when

measure of prothrombin since considerable free thrombin occurs in the serum. The conversion of thrombin to metathrombin is so rapid, however, that thrombin becomes a negligible factor in subsequent determinations

*Equipment and Reagents:*

The same as for Test 8, plus

Deprothrombinized rabbit plasma (See Test 9)

*Procedure* —Blood is obtained by venipuncture and exactly 2 cc. transferred to each of 3 test tubes These are placed in a water bath kept at 37° C Fifteen minutes after clotting has occurred, the first tube is centrifuged in a Clinical centrifuge for 1 minute at top speed After 30 seconds, the time required to stop the centrifuge, the prothrombin is determined as follows 0 1 cc of the serum is added to a mixture of 0 1 cc. deprothrombinized rabbit plasma, 0 1 cc thromboplastin and 0 1 cc. of 0 02 M calcium chloride The stop watch is clicked as the serum is blown in, and the time required for the formation of a clot is recorded The technique is the same as for the one-stage prothrombin method. Fifteen minutes later, the second tube is centrifuged in the same manner as the first, and the prothrombin time of the serum determined. The prothrombin of the first serum is again determined At 45 minutes the prothrombin time of both tubes is repeated At the end of the hour, the third tube is centrifuged and the prothrombin in the serum of each tube determined

*Application and Interpretation* —Just as the one-stage prothrombin test is a quantitative measure of the prothrombin complex and, with certain precautions, specifically of prothrombin itself, so the prothrombin consumption test is the determinant of the factors that produce available thromboplastin, and can be made to measure thromboplastinogen

By means of the prothrombin consumption test, it was found that only a very small amount of prothrombin is consumed when hemophilic blood clots The test is the first specific laboratory means for diagnosing hemophilia As the result of this test, the defect in hemophilic blood was established to be a lack of thromboplastinogen, and further, that the degree of deficiency is apparently constant from the time of birth and probably remains fixed throughout life In severe hemophilia only a trace of thromboplastinogen is present in the blood, while in mild cases the amount may be sufficient to meet ordinary emergencies By adding normal blood or plasma to hemophilic blood, the prothrombin consumption is increased because the lack of thromboplastinogen is partly corrected Since this is of great practical importance, this subject will be treated separately as Test 13

When the consumption of prothrombin is very low, the serum will

## Test 11

## THE PROTHROMBIN CONSUMPTION TEST

*Definition* —The quantitative determination of the prothrombin remaining in the serum after the blood has coagulated under standardized conditions constitutes the prothrombin consumption test.

*Principle.*—Present available evidence indicates that prothrombin, labile factor, calcium and thromboplastin interact stoechiometrically to form thrombin Normally, labile factor and calcium are in excess, therefore, the amount of prothrombin converted or consumed depends on the quantity of available thromboplastin The production of reactive thromboplastin involves a complex set of reactions Blood contains only inactive thromboplastinogen, which requires for its activation, an agent derived from platelets, but platelets fail to supply this activator until they are labilized by thrombin. This is the basis of the chain reaction, *i.e.*, the thrombinogenic cycle.

As blood clots in a test tube, the thrombin is rapidly adsorbed by the fibrin reticulum of the clot, therefore, the chain reaction is held in check, the platelets' disintegration is delayed, and only a trace of thromboplastinogen is activated This necessitates that the coagulated blood be centrifuged to separate the serum from intimate contact with fibrin surface in order that the chain reaction can proceed The optimum time to centrifuge after the clot has formed varies with different bloods It has been found that centrifuging one tube 15 minutes after coagulation, the second in 30 minutes and the third after 1 hour yields results which form a characteristic and informative pattern

During the reaction involving the activation of thromboplastinogen and its interaction with free prothrombin, another important change occurs, namely, prothrombinogen is being converted to free prothrombin The prothrombin consumption is therefore the result of several reactions some of which are independent of each other and occur simultaneously and concurrently

The prothrombin remaining in serum is determined by adding 0 1 cc of the serum to a mixture of equal volumes of deprothrombinized rabbit plasma, thromboplastin and 0 02 M calcium chloride. The calcium phosphate-treated rabbit plasma supplies both fibrinogen and a constant excess of labile factor

The value obtained immediately after centrifugation is not a true

measure of prothrombin since considerable free thrombin occurs in the serum. The conversion of thrombin to metathrombin is so rapid, however, that thrombin becomes a negligible factor in subsequent determinations.

*Equipment and Reagents:*

The same as for Test 8, plus:

Deprothrombinized rabbit plasma (See Test 9).

*Procedure.*—Blood is obtained by venipuncture and exactly 2 cc transferred to each of 3 test tubes These are placed in a water bath kept at 37° C. Fifteen minutes after clotting has occurred, the first tube is centrifuged in a Clinical centrifuge for 1 minute at top speed After 30 seconds, the time required to stop the centrifuge, the prothrombin is determined as follows 0 1 cc of the serum is added to a mixture of 0 1 cc. deprothrombinized rabbit plasma, 0 1 cc thromboplastin and 0 1 cc. of 0 02 M. calcium chloride The stop watch is clicked as the serum is blown in, and the time required for the formation of a clot is recorded The technique is the same as for the one-stage prothrombin method Fifteen minutes later, the second tube is centrifuged in the same manner as the first, and the prothrombin time of the serum determined. The prothrombin of the first serum is again determined. At 45 minutes the prothrombin time of both tubes is repeated. At the end of the hour, the third tube is centrifuged and the prothrombin in the serum of each tube determined

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The prothrombin remaining in serum is determined by adding 0 1 cc of the serum to a mixture of equal volumes of deprothrombinized rabbit plasma, thromboplastin and 0 02 M. calcium chloride. The calcium phosphate-treated rabbit plasma supplies both fibrinogen and a constant excess of labile factor

The value obtained immediately after centrifugation is not a true

ing platelets to plasma deficient in these cells, the prothrombin consumption can be made normal, as illustrated by the results presented in table 13.

In afibrinogenemia the prothrombin consumption proceeds rapidly as is to be expected because no fibrin is present to adsorb thrombin, thus permitting the chain reaction to be promptly initiated and to run its course rapidly as the following results illustrate:

| | | | | | | |
|---|---|---|---|---|---|---|
| Time after putting native plasma in glass, in minutes | 0 | 3½ | 5 | 10 | 15 | 45 |
| Prothrombin consumption time, in seconds | 12 | 15 | 10 | 20 | 40 | 44 |

The blood was collected with a silicone-coated syringe and needle and transferred to a silicone-coated test tube immersed in an ice bath. The blood was centrifuged at 800 r p m. for 10 minutes Two cc of the platelet-rich plasma was transferred to a glass test tube which was placed in a water bath kept at 37° C In carrying out the prothrombin consumption test, deprothrombinized human plasma was used as the source of fibrinogen The experiment was performed at *the Boston City Hospital through the courtesy of Dr. William Castle* and Dr F. H L Taylor

In marked hypoprothrombinemia, the prothrombin consumption should theoretically be nearly complete This is actually the case in [illegible]

thrombin consumption pattern is obtained which is not very unlike that of normal blood This is easily explained When the total prothrombin is low, nearly all is consumed during coagulation, whereas

TABLE 14 —PROTHROMBIN CONSUMPTION IN CONGENITAL HYPOPROTHROMBINEMIA AND AFTER THE ADMINISTRATION OF DICUMAROL

| Subject | *Type of hypoprothrombinemia* | *Prothrombin time sec* | *Tube* | *Minutes after the formation of a solid clot* 15 | 30 | 45 | 60 |
|---|---|---|---|---|---|---|---|
| | | | | *Prothrombin consumption time in seconds* | | | |
| B B | Free and prothrombinogen diminished Congenital | 19 | 1 | 23 | 155 | ∞ | ∞ |
| | | | 2 | | 19 | 154 | 225 |
| | | | 3 | | | | 54 |
| R F | Free diminished, prothrombinogen normal Congenital | 16 | 1 | 19 | 30 | 36 | 36 |
| | | | 2 | | 12 | 36 | 36 |
| | | | 3 | | | | 27 |
| M N | Free and prothrombinogen diminished Dicumarol 10 | 22 | 1 | 17 | 43 | 50 | 49 |
| | | | 2 | | 31 | 44 | 45 |
| | | | 3 | | | | 33 |

have a prothrombin time of about 8 seconds, whereas the prothrombin time of plasma is 12 seconds This is due to the fact that in serum all of the prothrombinogen has been converted to prothrombin while almost none of the latter has been activated to thrombin. As a result, the serum contains very much more free prothrombin than the original plasma

The prothrombin consumption test when carefully carried out is a quantitative measure of the hemophilic defect. Thus, a prothrombin consumption time of 8 seconds indicates a severe or almost complete lack of thromboplastinogen, whereas a value of 12 seconds suggests a relatively mild deficiency In the latter condition, the prothrombin consumption time of the plasma rich in platelets (obtained by slow centrifugation and using silicone glassware) is often more satisfactory and reliable than the value obtained on whole blood The prothrombin consumption of native platelet-rich plasma therefore is recommended for studying and diagnosing border-line cases The prothrombin consumption time is a quantitative measure *of the concentration of thromboplastinogen only if the total prothrombin is normal.* It is, therefore, essential in precise studies, to determine the total prothrombin (test 12) and if it is found not to be normal, to give the patient natural vitamin $K_1$ (2 capsules of Klotogen Abbott daily for 4 days) and then repeat the determination

When the test was developed, it was found that the removal of platelets brought about a very defective consumption of prothrombin [1] It was concluded that the same would occur in clinical thrombocytopenia This was later found to be true by Soulier[2] and by the writer and his associates [3] By means of the test it was established that a genuine coagulation defect is present in thrombocytopenia By add-

TABLE 13 —EFFECT OF ADDING PLATELETS TO THROMBOCYTOPENIC BLOOD ON THE PROTHROMBIN CONSUMPTION TIME

Subject Boy aged 8 years with chronic thrombocytopenia
Platelet count 65 000
Prothrombin time 12½ sec
Prothrombin consumption

| | *Minutes after formation of a solid clot* | | | |
|---|---|---|---|---|
| *Tube* | 15 | 30 | 45 | 60 |
| | *Prothrombin consumption time in seconds* | | | |
| 1 | 6 | 9½ | 9½ | 9½ |
| 2 | | 8 | 10 | 10 |
| 3 | | | | 9½ |
| 4* | 21 | 21 | 22½ | 22 |

* Washed platelets from 5 cc of normal blood were added to 2 cc. of the patient's blood

## Test 12

## DETERMINATION OF TOTAL PROTHROMBIN

*Principle*.—When human blood is collected in silicone-coated glassware, and subjected to high centrifugation to remove the platelets, a plasma is obtained in which little prothrombin is activated to thrombin because the platelet activator of thromboplastinogen is lacking. When such plasma is transferred to a glass test tube and incubated at 37° C., the prothrombinogen is converted to free prothrombin. Since very little prothrombin is consumed, the serum will contain almost all of the total amount of prothrombin in the free form, which can be determined by the prothrombin consumption procedure (Test 11).

*Apparatus and Reagents*.—Same as for Test 11.

*Procedure*.—Blood is collected by venipuncture with a silicone-coated syringe and needle, and immediately transferred to small silicone-coated test tubes which are immersed in an ice bath. After the blood is chilled for 10 minutes, the tubes are centrifuged at 8000 r.p.m. in an angle centrifuge for 10 minutes. One cc. of the clear plasma is removed with special care not to disturb the platelet layer and transferred to a small glass test tube which is placed in a water bath. As fibrin appears, it is removed by wrapping it about a glass stirring rod. As soon as the formation of fibrin is complete, 0.1 cc. of the serum is tested for its prothrombin concentration according to the technique of Test 11. The test is repeated every 5 minutes until a constant prothrombin consumption time value is obtained. This usually occurs in 10 to 15 minutes. The normal total prothrombin time value is 8 to 9 seconds. A typical result is as follows:

| *Time after coagulation* | *Prothrombin consumption time* |
|---|---|
| 5 min. | 7 sec. |
| 10 min. | 8 sec. |
| 15 min. | 8 sec. |
| 30 min. | 8 sec. |

*Application and Interpretation*.—As the result of the prothrombin consumption test, it was established that human blood contains much more inactive prothrombin than active. It can be calculated that human plasma contains approximately 3 units of prothrombinogen and 1 unit of free or active prothrombin, or, in other words, the active prothrombin constitutes only about one-fourth of the total.

if only the free is below normal, the total is not markedly reduced since the concentration of prothrombinogen is several times that of free prothrombin Therefore, some prothrombin remains after the thrombinogenic reaction is completed In the hypoprothrombinemia induced by Dicumarol, results similar to the second type of congenital hypoprothrombinemia are obtained This is due to the fact that the free prothrombin is more depressed than the total

A number of possibilities of the prothrombin consumption test have not been explored Its probable application for detecting a thrombotic tendency is well worthy of consideration. Preliminary studies make it appear likely that the test may become useful to detect qualitative deficiencies in platelets Like the one-stage pro-

TABLE 15 —CHANGE IN PROTHROMBIN CONSUMPTION TIME INDUCED BY SUBSTITUTING NORMAL PLATELETS FOR FUNCTIONALLY DEFICIENT PLATELETS

Subject Female aged 53 years with chronic myelogenous leukemia.
Platelet count. 657,000 (of plasma)
Prothrombin time 12 seconds
Prothrombin consumption.

| *Tube* | *Minutes after formation of a solid clot* 15 | 30 | 45 | 60 |
|---|---|---|---|---|
| | *Prothrombin consumption time in seconds* | | | |
| 1 | 9 | 12 | 13 | 14 |
| 2 | | 11 | 12½ | 14 |
| 3 | | | | 12 |
| 4* | 13 | 12½ | 14 | 14 |
| 5** | 28 | 28 | 27½ | 28 |

* Test done on native plasma obtained by slow centrifugation This plasma contained 657,000 platelets per cmm

** Test carried out on plasma from which platelets were removed by centrifugation, and an emulsion of normal dog platelets added

cedure for determining prothrombin, the prothrombin consumption test is a precise tool for research Indiscriminate attempts to modify the method will likely lead to the same disasterous results that befell the one-stage test Already such modifications are appearing. It seems not unreasonable to ask that any one who attempts to change or to modify the procedure should show evidence that he has thoroughly mastered the original technique, and that he should show wherein the proposed modification is superior to the basic method

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## Test 12

## DETERMINATION OF TOTAL PROTHROMBIN

*Principle.*—When human blood is collected in silicone-coated glassware, and subjected to high centrifugation to remove the platelets, a plasma is obtained in which little prothrombin is activated to thrombin because the platelet activator of thromboplastinogen is lacking. When such plasma is transferred to a glass test tube and incubated at 37° C., the prothrombinogen is converted to free prothrombin Since very little prothrombin is consumed, the serum will contain almost all of the total amount of prothrombin in the free form, which can be determined by the prothrombin consumption procedure (Test 11).

*Apparatus and Reagents* —Same as for Test 11.

*Procedure* —Blood is collected by venipuncture with a silicone-coated syringe and needle, and immediately transferred to small silicone-coated test tubes which are immersed in an ice bath After the blood is chilled for 10 minutes, the tubes are centrifuged at 8000 r p m in an angle centrifuge for 10 minutes One cc. of the clear plasma is removed with special care not to disturb the platelet layer and transferred to a small glass test tube which is placed in a water bath As fibrin appears, it is removed by wrapping it about a glass stirring rod As soon as the formation of fibrin is complete, 0 1 cc of the serum is tested for its prothrombin concentration according to the technique of Test 11 The test is repeated every 5 minutes until a constant prothrombin consumption time value is obtained This usually occurs in 10 to 15 minutes The normal total prothrombin time value is 8 to 9 seconds A typical result is as follows

| *Time after coagulation* | *Prothrombin consumption time* |
|---|---|
| 5 min | 7 sec |
| 10 min. | 8 sec |
| 15 min | 8 sec |
| 30 min | 8 sec |

*Application and Interpretation* —As the result of the prothrombin consumption test, it was established that human blood contains much more inactive prothrombin than active It can be calculated that human plasma contains approximately 3 units of prothrombinogen and 1 unit of free or active prothrombin, or, in other words, the active prothrombin constitutes only about one-fourth of the total

The physiological purpose of prothrombinogen is entirely unknown. In dog and rabbit blood almost all of the prothrombin is in the active form. As the result of preliminary studies, it does not appear likely that prothrombinogen functions directly in hemostasis. How the organism converts prothrombinogen to the reactive state remains unexplained. In view of the fact that prothrombinogen when stored in glass is completely changed to prothrombin, makes it appear likely that the inactive compound is prothrombin combined with an inhibitor which under certain conditions such as contact with glass is readily split off

The determination of total prothrombin has practical value. In Dicumarol therapy, the free prothrombin drops promptly, but it requires a number of days before the total is significantly affected[1] This undoubtedly accounts for the observation that the prothrombin as measured by the one-stage test, drops faster than when measured by the two-stage procedure since the latter test not only determines free but also part of the prothrombinogen which is changed to the active form due to the various manipulations

The determination of total prothrombin is important in differentiating the two types of true congenital hypoprothrombinemia In one type the free is low, but the total is practically normal, whereas in the second type both free and inactive prothrombin are diminished

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## Test 13

### ASSAY OF THROMBOPLASTINOGEN

*Definition.*—Thromboplastinogen is a plasma constituent which is converted by a factor derived from platelets to active thromboplastin.

*Principle.*—In severe hemophilia the thromboplastinogen level of the blood is exceedingly low as shown by the extremely poor prothrombin consumption when such blood clots. Hemophilic blood or plasma therefore is a suitable assay medium for determining the thromboplastinogen content of non-hemophilic blood The hemophilic blood is mixed with the blood to be tested in varying proportions and the mixture is allowed to coagulate under standardized conditions as outlined for the prothrombin consumption test, and the prothrombin remaining is determined by the latter procedure

*Apparatus and Reagents*—The same as for Test 11

*Procedure*—Hemophilic blood is collected by means of a silicone-coated syringe and needle, and is immediately transferred to a silicone-coated test tube immersed in an ice bath About 15 to 20 cc. of blood are required The blood to be tested is collected in the same manner The mixtures are made by means of serological pipettes coated with silicone The proportions of hemophilic to normal blood in the mixtures tested are 9 to 1, 4 to 1, and 1 to 1

*Application and Interpretation*—The assay method outlined for thromboplastinogen is the only reliable method at present available to test the efficacy of blood or plasma for correcting the defect in hemophilic blood The test is important clinically, although it probably is not always a true quantitative measure of thromboplastinogen In the prothrombin consumption determination on which the test is based a number of factors participate, and therefore the amount of thromboplastin made available, rather than the thromboplastinogen, is actually measured Under carefully controlled conditions and with an adequate supply of platelets, nearly all the thromboplastinogen becomes available thromboplastin

Clinically it is important that the correction of the thromboplastinogen deficiency brought about by adding normal to hemophilic blood is quantitatively demonstrable both in vitro and in vivo In tables 5 and 6 the efficacy of normal blood to increase the prothrombin consumption of hemophilic blood when added in vitro is demonstrated It is therefore possible to determine the potency of the blood

before it is given, and then to check its efficacy in vivo. The results recorded in table 16 are instructive. Immediately after the adminis-

TABLE 16—THE EFFECT OF GIVING NORMAL PLASMA INTRAVENOUSLY ON THE PROTHROMBIN CONSUMPTION TIME OF THE BLOOD OF A HEMOPHILIAC

| | *Clotting time Lee White* | *Tube* | *Minutes after the formation of a solid clot* 15 | 30 | 45 | 60 |
|---|---|---|---|---|---|---|
| | | | *Prothrombin consumption time in seconds* | | | |
| Before treatment | 60 min | 1 | 8 | 8 | 8 | 8 |
| | | 2 | | 8 | 8 | 8 |
| | | 3 | | | | 8 |
| After 500 cc. of plasma | 9 min. | 1 | 6 | 15 | 14 | 14 |
| | | 2 | | 8 | 14½ | 15 |
| | | 3 | | | | 13 |
| Twenty-four hours later | 8 min | 1 | 7 | 10 | 10 | 10 |
| | | 2 | | 10 | 10 | 10 |
| | | 3 | | | | 10 |
| Twelve hours after 500 cc of blood | 10½ min | 1 | 7 | 11 | 11 | 11 |
| | | 2 | | 9 | 9 | 9 |
| | | 3 | | | | 9½ |

tration of 500 cc. of plasma (24 hrs. old.) the prothrombin consumption time of the hemophilic blood was increased from 8 to 15 seconds. This permitted the extraction of two upper third molars. No abnormal bleeding occurred. In 24 hours the prothrombin consumption time dropped to 10 seconds This indicated the need of an additional transfusion, and 500 cc of fresh blood were given No bleeding occurred and the following morning the prothrombin consumption time was 11 seconds No further treatment was given Thus, with 500 cc of plasma and 500 cc of blood, together with careful laboratory control by means of the prothrombin consumption test, two molars were extracted with no abnormal bleeding. It will be observed that the clotting time failed to correspond to the prothrombin consumption test and was misleading

## Test 14

## PREPARATION AND ASSAY OF THROMBIN

*Principle.*—As the result of studies by Mellanby, Eagle and others, it was found that when plasma is diluted with cold distilled water and is acidified, a precipitate is formed which when dissolved and neutralized, yields thrombin and fibrin on the addition of calcium On removing the fibrin, a relatively potent and fairly stable solution of thrombin is obtained The composition of the precipitate formed when diluted plasma is acidified is not known. It is fairly certain that in addition to prothrombin and fibrinogen, it contains labile factor and thromboplastin. Curiously, a much more potent preparation of thrombin can be obtained from human than from dog or rabbit plasma In fact, by rigidly following the standard technique as outlined, human plasma yields a preparation of thrombin which is *remarkably constant in its potency*. Such a preparation has been employed in the writer's laboratory as a standard and designated "full strength" (F.S ) since 1936[1] In terms of the unit which was later adopted, 1 cc contains approximately 50 units Although commercial thrombin is now available, the method for preparing thrombin is so simple and inexpensive that it should be familiar to all students of coagulation

The assay of thrombin depends on the speed with which it clots a standard solution of fibrinogen or fresh plasma If the clotting times are plotted against the concentration of thrombin, a hyperbolic curve is obtained (Fig 12 ) with the asymptodes zero and infinity, *i e*, at infinite dilution of thrombin, the clotting time is infinity and at high concentration, instantaneous The segment of the curve presented in figure 12 is for practical purposes the most important It can be expressed by the equation·

$$ct = a + \frac{k}{C} \qquad ct = \text{clotting time}$$
$$C = \text{concentration of thrombin}$$
$$a \text{ and } k = \text{constants}$$

This can be considered a fundamental law of blood clotting since nearly all the quantitative procedures employed for determining the various agents taking part in the clotting reactions depend on it either directly or indirectly Although fibrinogen is the ideal testing medium for thrombin, it has certain disadvantages Unless its concen-

tration, salt concentration and pH are carefully controlled, unreliable results will be obtained. It is therefore more convenient and simpler to use fresh oxalated human plasma as the assay medium since the variables are automatically controlled by the constancy of its composition.

*Apparatus and Reagents:*

Centrifuge.

Cylinder containing carbon dioxide. If not available, carbon dioxide may be generated by dropping hydrochloric acid on calcium carbonate

Sodium bicarbonate, 5 per cent solution

Calcium chloride 0.1 M.

*Preparation of Thrombin.*—In a 250 cc. beaker immersed in an ice bath, 100 cc of distilled water and 10 cc. of fresh oxalated or citrated plasma are mixed. When the solution is cooled to 2° C., a slow stream of carbon dioxide is bubbled through for 10 minutes. The precipitate which is formed is removed by centrifugation. It is convenient to use four 15 cc conical centrifuge tubes These are centrifuged, the clear liquid poured off, and more of the reaction mixture added. When all of the precipitate is packed, the tubes are drained, and the walls carefully dried with filter paper rolled to fit the inside of the tube. To each of the 4 tubes, 2 cc of 0.85 per cent sodium chloride and 0.1 cc of 5 per cent sodium bicarbonate are added. The tubes are stirred until the precipitate is dissolved The solutions are combined and neutralized to approximately pH 7 by adding a few drops of sodium bicarbonate and testing with a universal indicator paper. Enough saline solution is added to bring the volume to 10 cc It is put in a water bath and 0.1 cc of 0.1 M. calcium chloride is added The clot which forms in 45 to 120 seconds is removed by wrapping it about a glass stirring rod The thrombin solution thus prepared is water clear, and is constant in potency It is designated full strength (F S) *When this solution is frozen and kept in the freezing compartment* of a refrigerator, its full potency is preserved for months

TABLE 17.—THE RELATION OF THE CONCENTRATION OF THROMBIN TO THE CLOTTING TIME

| *Strength* | *Full* | $\frac{1}{2}$ | $\frac{1}{5}$ | $\frac{1}{10}$ | $\frac{1}{20}$ | $\frac{1}{40}$ | $\frac{1}{100}$ | $\frac{1}{200}$ |
|---|---|---|---|---|---|---|---|---|
| Concentration of thrombin* | 100 | 50 | 20 | 10 | 5 | 2.5 | 1 | 0.5 |
| Number of units per cc.** | 50 | 25 | 10 | 5 | 2.5 | 1.25 | 0.5 | 0.25 |
| Clotting time in seconds*** | 3 | 4 | 7 | 11 | 18 | 33 | 80 | 180 |

* The dilutions are made with distilled water

** The values are only approximate

*** The determination is as follows 0.1 cc of the thrombin solution is blown into 0.2 cc of fresh oxalated human plasma and the clotting accurately timed at 37° C

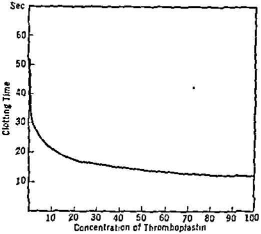
*Assay of Thrombin.*—Two-tenths cc of fresh oxalated human plasma are measured into a test tube, and after warming it in the water bath for 15 seconds, 0 1 cc of the thrombin solution to be tested is blown in. The time required for a clot to form is carefully determined by means of a stop watch The result is compared with the values given in table 17. The data in this table were obtained with standard full strength thrombin which was progressively diluted.

With the values of table 16 as reference, the thrombin concentration of any unknown solution can readily be determined If the clotting time is between 3 and 180 seconds, the concentration can be read directly from the curve constructed from the above values If a clotting time less than 3 seconds is obtained, the solution is diluted with distilled water until a concentration is reached which, when tested according to the described method, *i e*, 0 1 cc of the thrombin solution added to 0 2 cc. of human plasma, yields a clotting time within the range of the curve.

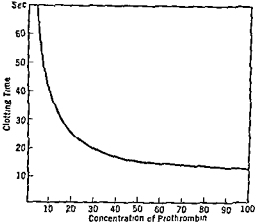
As an example to illustrate the calculation An ampule of 5000 units of commercial thrombin dissolved in 5 cc of physiological saline was tested as follows Various dilutions with distilled water were made The clotting time was less than 3 seconds until a 1 to 20 dilution was prepared (0 1 cc diluted with 1 9 cc distilled water) This dilution gave a clotting time of exactly 3 seconds and the $\frac{1}{5}$, $\frac{1}{10}$, $\frac{1}{20}$ and $\frac{1}{40}$ dilution of this solution gave clotting times of $7\frac{1}{2}$, 12, 19 and 38 seconds respectively It is obvious therefore that the original solution (the 5 cc. containing 5000 units) is 20 times F S

*Application and Interpretation* —The principal use of thrombin as a laboratory reagent is for the assay of heparin This application is discussed in detail under Test 15 The assay of thrombin preparation intended for therapeutic purposes such as topical application is rarely necessary as highly potent preparations are available commercially

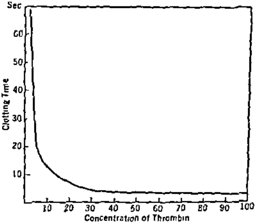
While the practical applications of thrombin are limited, its theoretical importance deserves emphasis The curve correlating clotting time with the concentration of thrombin underlies the whole structure of the coagulation mechanism This is illustrated for instance by the marked similarity of the prothrombin and the thromboplastin curves to the thrombin curve (Fig 12) In the prothrombin curve all the variables except prothrombin are maintained constant, therefore the amount of thrombin formed is directly dependent on the concentration of prothrombin In the thromboplastin curve, the thromboplastin is the variable, while the other agents (prothrombin, labile factor and calcium) are fixed The thromboplastin therefore is the determinant of the amount of thrombin formed Similar curves are obtained when the labile factor and calcium are made the variables



Sec
60
50
40
30
20
10
Clotting Time
10 20 30 40 50 60 70 80 90 100
Concentration of Thromboplastin

Sec
60
50
40
30
20
10
Clotting Time
10 20 30 40 50 60 70 80 90 100
Concentration of Prothrombin

Sec
60
50
40
30
20
10
Clotting Time
10 20 30 40 50 60 70 80 90 100
Concentration of Thrombin

It is difficult to see how these curves can be explained on any basis other than that the reaction:

Prothrombin + labile factor + calcium + thromboplastin = thrombin

is stoechiometric. When any three of the primary agents are made constant the amount of thrombin formed is determined by the fourth which is made a variable.

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## Test 15

## THE QUANTITATIVE ESTIMATION OF FIBRINOGEN

*Principle* —For the determination of fibrinogen in blood or plasma, two steps are required The first is the complete removal of the compound from the plasma, while the second step is its actual estimation Fibrinogen is quantitatively precipitated by half-saturated sodium chloride, one-quarter saturated ammonium sulfate or 12½ per cent sodium sulfite [1] It can also be converted to fibrin and removed as a clot. The latter is the basis of the widely used procedure of Cullen and Van Slyke in which oxalated plasma is diluted with saline solution and an excess of calcium chloride is added. The actual estimation of the fibrin can be made by drying and weighing it, or determining it from its nitrogen or tyrosine content

The Cullen and Van Slyke procedure was selected for the routine estimation of fibrinogen, since it determines specifically the amount of the compound which is coagulable, *i e*, the fraction which is true fibrinogen The salting out methods have the disadvantage that they may precipitate other proteins One important modification was made, namely, the plasma is diluted with distilled water instead of saline solution.[2] The yield of fibrin is thereby increased approximately 20 per cent It appears that the higher value is the more correct one since it corresponds fairly closely with the quantity of fibrinogen obtained by precipitation with half-saturated sodium chloride The fibrin obtained is conveniently estimated by its content of tyrosine To do this the fibrin is hydrolyzed by boiling with sodium hydroxide solution, and determining the tyrosine by means of Folin-Ciocalteu phenol reagent The factor to convert tyrosine to fibrin is 10 7 It was obtained by carefully checking the ratio of nitrogen to tyrosine in both purified fibrinogen and fibrin

*Apparatus and Reagents*

Coleman junior Spectrophotometer
Cuvette
Centrifuge
Test tubes
Calcium chloride 0 1 M
Sodium hydroxide 10 per cent.
Sodium carbonate 20 per cent.

Phenol reagent (Folin-Ciocalteu). Into a 1500 cc. Florence flask 100 mg. sodium tungstate, 25 gm. sodium molybdate, 700 cc. distilled water, 50 cc. of 85 per cent phosphoric acid and 100 cc of concentrated hydrochloric acid are added. The solution is refluxed gently for 10 hours. To this 150 gm. of lithium sulfate, 50 cc. of water and a few drops of bromine are added. The excess bromine is removed by boiling The solution is cooled and diluted to 1 liter. If the reagent develops a green color, it is again treated with bromine and boiled.

Tyrosine standard stock solution Two hundred mg of pure tyrosine are dissolved in 1000 cc. of 0.1 N. hydrochloric acid. 1 cc. = 0 2 mg tyrosine.

*Procedure.*—One cc. of oxalated plasma is diluted with 25 cc of distilled water in a 30 cc. beaker, and 2 cc of 0 1 M. calcium chloride are added. After 30 minutes the voluminous clot is removed by wrapping it onto a stirring rod. It is thoroughly washed with distilled water, then removed from the glass rod and put into a glass test tube The clot is digested by covering it with 0 5 cc. of 10 per cent sodium hydroxide and heating it in a boiling water bath for 10 minutes

The alkaline solution is diluted in a volumetric flask to 25 cc. A portion of the solution is centrifuged to remove suspended calcium oxalate, and 5 cc of the centrifuged solution is transferred to a test tube (15 × 250 mm ) Then 4 5 cc of distilled water, 0 5 cc of phenol reagent and 3 cc of 20 per cent sodium carbonate are added After mixing by inversion, the reaction mixture is allowed to stand 30 minutes for a maximum color development. The color intensity is measured in a Coleman junior Spectrophotometer at 560 mu The value obtained is read directly in terms of tyrosine or fibrinogen from a curve prepared as follows a series of test tubes containing 0 02, 0 04, 0 06, 0 08 and 0 1 mg tyrosine (0 1, 0.2, 0 3, 0.4 and 0 5 cc of the stock tyrosine solution) are diluted to 9 5 cc with distilled water To each tube 0 5 cc. of phenol reagent and 3 cc. of 20 per cent sodium carbonate are added After 30 minutes, the intensity of the color in the 5 tubes is read in the spectrophotometer at 560 mu From the values obtained a curve is constructed on semi-logarithmic graph paper.

The determination of fibrinogen in plasma lacking sufficient potential thrombin of its own, requires the addition of extra thrombin One cc of plasma is diluted with 25 cc of distilled water, and 2 cc. of 0 1 M. calcium chloride and 0 3 cc thrombin (F S , *see* Test 14) are added

*Application and Interpretation* —Fibrinogen is the passive agent in the coagulation reaction Normally its concentration varies from 250 to 400 mg. per 100 cc of plasma but it is readily increased by various stimuli In infections, it may be increased to 1000 mg or

more. It is generally above normal postoperatively, and is elevated in the latter period of pregnancy. The physiological significance of these marked changes in the fibrinogen level is not known, but it is certain that they have little, if any, influence on or relation to hemostasis It is to be emphasized that the higher the fibrinogen concentration, the less marked is clot retraction. From this one can conclude that the increase in the fibrinogen level protects from rather than predisposes to venous thrombosis. (*See* Chapter 5.)

How low the fibrinogen level must sink before defective hemostasis manifests itself is difficult to state since even in complete afibrinogenemia the bleeding tendency may be mild. With the exception of a few reported cases of hypofibrinogenemia, no information is available on the incidence of congenital low fibrinogen, nor is it known whether stimuli which increase the fibrinogen in normal subjects, are effective in such cases

In severe liver damage, the fibrinogen is decreased, but since both prothrombin and labile factor are usually also diminished, it is certain that the bleeding observed in acute hepatitis or yellow atrophy is not due primarily to hypofibrinogenemia

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## Test 16

### ASSAY OF HEPARIN

*Principle.*—The satisfactory assay of heparin is difficult because its anticoagulant action is complex It is an antiprothrombin, *i e*, it reacts with prothrombin and thereby prevents the activation of this compound to thrombin. Heparin likewise is a powerful antithrombin, but for this action it requires a co-factor, a compound present in the albumin fraction, which has been named Albumin X (*See* Fig 9). Heparin alone does not inactivate thrombin Since heparin with its co-factor removes thrombin practically instantaneously, it prevents the agglutination and disintegration of platelets It thereby exerts a third type of inhibitory action on clotting Heparin has no significant antithromboplastic action, and it should be emphasized that thromboplastin does not neutralize or inactivate heparin

Heparin can be assayed by its inhibitory effect on the clotting time of blood or of recalcified plasma This measures its over-all anticoagulant action Its potency or concentration can also be measured by its antithrombic activity For this a known quantity of thrombin standardized on normal plasma is added to plasma containing varying amounts of heparin to be tested The prolonged clotting time is a direct measure of heparin activity No test is known whereby heparin potency can specifically be estimated on the basis of its antiprothrombic action, but a method in which this property probably is a factor is in the prothrombin time test When increasing amounts of heparin are added to plasma, its prothrombin time becomes progressively prolonged and this action can be used as a quantitative measure of heparin

The assay of heparin by its effect on the clotting time is historically important because by this means Howell and later the Toronto group isolated a highly purified heparin Unfortunately the methods based on the clotting time are laborious and subject to numerous errors because the various factors upon which the clotting time depends cannot be completely controlled By employing plasma and determining the clotting time after recalcification, the procedure is simplified but it still retains the disadvantage of many uncontrollable variables, particularly the lack of constancy of available thromboplastin

In 1936 the writer[1] developed two types of assay which meet the requirements of simplicity, accuracy and reliability They are

applicable to the determination of the heparin concentration in the blood, and also to the assay of the potency of commercial preparations The first method is based on standardizing the effect of varying quantities of heparin on the clotting time of plasma to which a thrombin of fixed potency is added For convenience this can be called the thrombin titration method Fresh oxalated human plasma is employed. The thrombin solution used for the test is of such strength that when 0 1 cc is added to 0 2 cc. of normal plasma, coagulation occurs in exactly 7 seconds By means of this test, concentrations of heparin ranging from 1 to 5 gammas per cc of plasma can be determined.

The second method is based on the effect of heparin on the prothrombin time of fresh oxalated human plasma With this procedure, heparin concentrations ranging from 5 to 25 gammas per cc of plasma are estimated. In both methods a highly purified commercial heparin serves as a standard In perfecting these methods, Abbott, La Roche-Organon, Lederle, Lilly and Upjohn brands of heparin were used These were generously supplied by the respective companies

*Apparatus and Reagents* —Same as for Test 8 plus a 0 1 cc serological pipette graduated in 0 001 cc

*Thrombin* ($\frac{1}{5}$ *S*) —The thrombin is prepared as described in Test 14 One part of thrombin (F S ) is diluted with 4 parts of distilled water If this dilution does not give a clotting time of 7 seconds when tested on fresh oxalated plasma, adjustments in the dilution must be made until the desired potency is obtained

*Procedure* —Thrombin titration method Commercial heparin (100 mg of the sodium salt in 10 cc ) is employed, and 0 25 cc of the solution transferred to a 50 cc volumetric flask and diluted with distilled water The diluted solution contains 50 gammas per cc or 0 01 cc = 0 5 gamma By means of a 0 1 cc serological pipette, 0 01 cc of the heparin solution is transferred to a test tube and mixed with 0 5 cc of fresh oxalated human plasma After 5 minutes of incubation at 25° C , 0 2 cc of the plasma is transferred to another test tube which is placed in a water bath (37° C ) for 15 seconds By means of a 1 cc serological pipette, in which the correctness of the last 0 1 cc , *i e*, from the tip to the first calibration mark has been carefully checked, 0 1 cc of thrombin ($\frac{1}{5}$ S ) is blown into the plasma, and the clotting time determined by means of a stop watch Considerable experience must be acquired to perform this test since the detection of the incipient clot, which is the end point, is difficult The test is repeated adding 0 02, 0 03, 0 04 and 0 05 cc of heparin to 0 5 cc of plasma A typical set of results is as follows

| Volume of heparin in cc * | 0 | 0 01(1)** | 0 02(2) | 0 03(3) | 0 04(4) | 0 05(5) |
|---|---|---|---|---|---|---|
| Clotting time in seconds | 7 | 9 | 12 | 16 | 22 | 30 |

* Added to 0 5 cc. of plasma

** The figures in parentheses are the number of gammas of heparin per cc of plasma

By selecting one preparation of heparin as the standard, other samples of heparin can be compared. The various commercial brands which were tested, showed a fair uniformity of potency, although an occasional sample was observed to vary as much as 20 per cent from the average.

In this test, it is important to exercise great care in collecting and preserving the oxalated plasma used as the assay medium Blood is obtained as in the prothrombin time method (Test 8) Silicone-coated glassware is used and the plasma is kept in a silicone-coated tube immersed in an ice bath Under these conditions the plasma remains constant in its response to heparin and thrombin for at least 3 hours

Assay of heparin by the prothrombin time test Commercial heparin (containing 100 mg of the sodium salt in 10 cc ) is employed Exactly 0 2 cc. of the solution is diluted with 3.8 cc of distilled water (1 cc = 500 gammas or 0 01 = 5 0 gammas of heparin) For the assay of heparin, 0 01 cc of the diluted heparin solution is mixed with 0 5 *cc of oxalated* human plasma collected with the same care as in the thrombin titration test After 5 minutes of incubation at 25° C , the prothrombin time is determined on 0 1 cc of the plasma in the usual manner (Test 8) A series of tests with 0 02, 0 03, 0.04 and 0 05 cc of heparin are carried out. The results with one brand of heparin were as follows

| Volume of heparin in cc * | 0 | 0 01(10)** | 0 02(20) | 0 03(30) | 0 04(40) | 0 05(50) |
|---|---|---|---|---|---|---|
| Prothrombin time in seconds | 12 | 18 | 25 | 54 | 70 | 120 |

*Added to 0 5 cc oxalated human plasma

** The figures in parentheses are the number of gammas of heparin added to 1 cc of plasma

The test is technically simpler than the thrombin titration test It does not require the meticulous adjustment of a standard thrombin solution to a precise potency nor is the end point (the incipient clot) as difficult to see The prothrombin time method is however less sensitive. It is best for the range of 10 to 50 gammas per cc of plasma

For the determination of heparin in human blood either the thrombin tritration or the prothrombin time method can be used If the concentration of heparin is low – less than 10 gammas per cc —the first procedure should be used. The prothrombin time test cannot be used for the determination of heparin in rabbit or dog blood because these

bloods have such a high prothrombin concentration that the quantity of thrombin formed far exceeds the heparin co-factor concentration

In determining the heparin content of plasma by the thrombin titration procedure, it is advisable to check whether the inhibitory activity is due to heparin. This can readily be done by adding protamine which neutralizes heparin. The following serves as an illustration observed in a patient:

| | |
|---|---|
| Patient's oxalated plasma | 0 2 cc |
| Thrombin (½ S ) | 0 1 cc |
| Clotting time | 30 sec |
| Normal oxalated plasma | 0 2 cc |
| Thrombin (½ S ) | 0 1 cc |
| Clotting time | 7 sec |
| Patient's oxalated plasma | 0 2 cc |
| Protamine 0 5 per cent | 0 01 cc |
| Thrombin (½ S ) | 0 1 cc. |
| Clotting time | 6 sec |

From these results, it can be concluded that the concentration of heparin in the patient's plasma was approximately 5 gammas per cc.; and that it actually was heparin since the antithrombic effect was abolished by protamine

*Application and Interpretation* —The quantitative determination of heparin in blood has clinical usefulness although heparinemia in man is exceedingly uncommon. While a hemorrhagic tendency can theoretically occur from an outpouring of heparin into the blood, this has apparently not been observed clinically Since marked heparinemia readily occurs in dogs after peptone or anaphylactic shock, one is inclined to speculate whether a similar liberation of heparin into the blood can occur occasionally in man during acute allergic attacks

The administration of heparin in the prophylaxis against thrombosis is generally controlled by the coagulation time A correlation of the coagulation time, the thrombin titration time and the prothrombin time after the injection of heparin can be seen in table 18.

TABLE 18 —EFFECT OF HEPARIN ON THE COAGULATION, THROMBIN AND PROTHROMBIN TIMES

Subject A Weight 57 kg
Heparin 3 cc (30 mg ) given intravenously

| *Time After Injection* min | *Coagulation Time* min | *Thrombin (½ S ) Time* sec | *Prothrombin Time* sec |
|---|---|---|---|
| 0 | 5½ | 7 | 12 |
| 5 | 40 | 60 | 19 |
| 15 | 35 | 35 | 16 |
| 30 | 30 | 29 | 15 |
| 60 | 24 | 22 | 13½ |

It will be observed that the correlation between the coagulation time, the thrombin titration, and the prothrombin time test is remarkably good The gradual decrease of the heparin concentration is shown by all three procedures.

For the establishment of an absolute heparin standard, it would be necessary to obtain the compound in a completely pure state, a possibility which according to authorities such as Jorpes[2] is very remote A provisional international heparin standard has been defined. It is an anhydrous salt of heparin prepared from the crystalline barium salt and supplied by the Connough Laboratories of the University of Toronto It is not clear whether the standard Toronto heparin is supplied as a substance of reference for the assay of the various brands now on the market.

Heparin potency can be defined in terms of its antithrombic activity as determined by the thrombin titration technique This requires a very carefully standardized solution of thrombin, and several specimens of normal oxalated human plasma to eliminate the effect due to differences in plasma. Since commercial brands of heparin having a high degree of potency are available, it is more expedient to select a brand which has a high and uniform activity as a standard of reference.

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2. Jorpes, J E *Heparin in the treatment of thrombosis Second Edition* London, Oxford University Press, 1946

## Test 17

# ASSAY OF ANTITHROMBIC ACTIVITY OF PLASMA

*Principle.*—Plasma contains a thermolabile substance which inactivates thrombin. This agent is generally known as the normal antithrombin of the blood. Since it is found in the albumin fraction, it has also been named Albumin X It acts by combining with thrombin and the resulting complex is called metathrombin. A roughly quantitative measure of the antithrombic activity of plasma can be made by determining the speed with which thrombin disappears when a fixed volume of the plasma to be tested is added to a standard amount of thrombin [1]

*Apparatus and Reagents* —Same as for Test 15.

*Procedure* —The plasma to be assayed for antithrombic activity is defibrinated as follows 0 1 cc. of thrombin (F S ) is added to 2 cc of plasma The fibrin is removed with a glass rod

To determine the antithrombic activity, 0 2 cc. of the defibrinated plasma is mixed with 0 8 cc. of thrombin (F S ) and the mixture incubated at 25° C. After one minute, the thrombic activity of the mixture is determined by removing 0 1 cc and adding it to 0 2 cc of fresh oxalated human plasma The time required to form a clot is accurately determined with a stop watch The reaction is carried on in a water bath at 37° C. The determination is repeated after 1, 5, 10, 15, 20 and 30 minutes.

A typical set of results are given in table 19.

TABLE 19 —THE ANTITHROMBIC ACTIVITY OF NORMAL HUMAN PLASMA

| *Subject* | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| *Time** | | *Clotting time†* | | |
| *min* | *sec* | *sec* | *sec* | *sec* |
| 0 | 3 | 3½ | 3½ | 3 |
| 1 | 4½ | 4 | 4½ | 4 |
| 3 | 5⅓ | 6 | 8 | 7 |
| 5 | 6½ | 8 | 10 | 11 |
| 10 | 9 | 11 | 12 | 17 |
| 15 | 11 | 13 | 14½ | 26 |
| 20 | 15 | 13 | 15 | 30 |
| 25 | 17½ | 17 | 18 | 34 |
| 30 | 18 | 21½ | 19 | 38 |

* Thrombin (F S ) 0 8 cc was mixed with 0 2 cc of the defibrinated plasma to be tested

† Thrombin potency was tested by adding 0 1 cc of the mixture to 0 2 cc of fresh oxalated human plasma

*Application and Interpretation* —Since fibrinogen has a greater affinity for thrombin than the normal antithrombin of the plasma, little thrombin is neutralized until all the fibrinogen is coagulated The plasma antithrombin (Albumin X) therefore does not prevent or delay clotting. Furthermore, as has been fully discussed in Chapter 5, fibrin itself appears to be the most important physiological antithrombin. It becomes doubtful therefore whether the normal plasma antithrombin has a primary function in preserving the fluidity of the blood While it does possess the power to neutralize large quantities of thrombin, it is difficult to conceive how this function is utilized physiologically.

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## Test 18

# DECALCIFICATION OF BLOOD WITH ION EXCHANGE RESIN

*Principle*.—Certain phenol-formaldehyde resins such as Amberlite IR-100 (Resinous Products and Chemical Co., Philadelphia) have the property when in the sodium phase to exchange that ion for calcium. Such a resin quantitatively removes calcium from blood as Steinberg first showed.[1] The introduction of silicone-coated glassware greatly improved the procedure[2] for it depresses coagulation and therefore permits decalcification without any formation of fibrin.

*Apparatus and Reagents*.—Amberlite tube. A cylindrical glass tube (8 mm. internal diameter, 15 cm. long) with a constricted end fitted with a cork and needle of a large bore (No. 15). *See* figure 14.

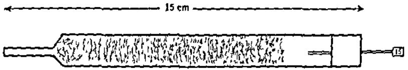



FIG. 14.—Amberlite tube. The inside of the tube is coated with silicone. A plug of glass wool also treated with silicone is inserted, and the tube filled with Amberlite. The cork and a No. 15 needle are likewise coated with silicone.

Purification of Amberlite IR-100 (Method of Stefanini[3]). Twenty grams of the resin mixed with 100 cc. of 5 per cent (by volume) sulfuric acid. The mixture is boiled for 5 minutes and stirred vigorously. The acid is poured off and the resin washed by decantation with distilled water until the wash water is no longer acid. The Amberlite is then treated with 100 cc. of 5 per cent sodium carbonate, heated to 70° C. and stirred. Much coloring matter is extracted. The solution is poured off and the process repeated until the sodium carbonate becomes only slightly colored, after which it is washed with distilled water until all traces of sodium carbonate have been removed.

The purified material is then mixed with 250 cc. of 5 per cent sodium chloride and stirred with a mechanical stirrer for 30 minutes. After standing 60 minutes or more, the resin is transferred to a Buchner funnel and washed by suction with distilled water until the filtrate is free of chlorine ions. It is dried at 37° C.

*Procedure*.—Blood is collected by venipuncture using a silicone-

coated syringe and needle. The syringe is kept in an ice bath prior to use. Ten cc. of blood is slowly passed through the tube containing 3 gm of Amberlite and collected in a silicone-coated test tube immersed in an ice bath. The blood is retransferred to the syringe and again passed through the Amberlite tube. To assure complete decalcification, the process is repeated a third time.*

The blood thus treated will not clot even when an excess of thromboplastin is added. No calcium is detectable and probably all magnesium of the plasma is also removed None of the other common constituents of the blood appear to be affected or removed The plasma usually is slightly colored

*Application and Interpretation* —Amberlite serves as a very satisfactory method to remove calcium from blood without adding an anticoagulant. It has been possible by this means to determine the exact amount of calcium required for coagulation. It offers a new approach to the study of calcium in the clotting reaction Interestingly, Amberlite readily removes both ionized and un-ionized or combined calcium from blood, which indicates that the un-ionized calcium cannot be tightly bound

Whether decalcification by means of exchange-ion resins will become practical to preserve blood for transfusions remains to be determined In the solution of this problem, it will not only require mastery of the technical difficulties and a decision as to the economy of the procedure, but also information as to whether the slight amount of resin which dissolves in the blood is harmless

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2 QUICK, A J On the quantitative relationship between calcium and prothrombin, Am J Physiol, 148, 211, 1947
3 STEFANINI, M Purification of the resin Amberlite IR-100 for blood coagulation studies, Proc Soc Exper Biol and Med, 67, 22, 1948

* The Amberlite may be recovered as follows Saline is passed through until all the blood is washed out It is then treated with 5 per cent sodium carbonate then distilled water and recharged with sodium chloride

## Test 19

## PROTHROMBIN ADSORBENTS

*Principle*—A number of insoluble salts and bases including tricalcium phosphate, barium sulfate, aluminum hydroxide, magnesium hydroxide, calcium fluoride, and calcium oxalate remove prothrombin from plasma by adsorption. These agents differ markedly, however, in their efficiency to deprothrombinize plasma, and also vary in their property of adsorbing other constituents of plasma such as fibrinogen and labile factor. Some adsorbents, particularly tricalcium phosphate and barium sulfate, do not remove prothrombin from citrated plasma, whereas aluminum hydroxide does. This finding is probably related to another observation, namely that sodium citrate quantitatively elutes prothrombin from tricalcium phosphate and barium sulfate, but only partially from aluminum hydroxide.

*Preparation of Adsorbent.**—Tricalcium phosphate 0 2 M. suspension (See Test 9.)

Barium sulfate 0.15 M. suspension. A solution of 36.6 gm barium chloride in 500 cc. of distilled water is slowly added with vigorous stirring to a solution of 8 3 cc. of concentrated sulfuric acid in 500 cc of distilled water. The reaction mixture is kept at 25° C. The precipitated barium sulfate is washed by decantation until free of chloride ions The volume is brought to 1 liter It is allowed to stand several days, before it is used since its adsorptive activity requires time to become stabilized

Aluminum hydroxide 0 2 M suspension. To a solution of 66 6 gm. of aluminum sulfate ($Al_2(SO_4)_3.18H_2O$) in 500 cc of distilled water, 500 cc of 5 per cent ammonium hydroxide are slowly added with vigorous stirring The gelatinous precipitate is washed with distilled water until the wash water is no longer alkaline. The suspension of aluminum hydroxide is brought to 1 liter

Magnesium hydroxide 0 2 M suspension To a solution of 49 5 gm of magnesium sulfate ($MgSO_4$ $7H_2O$) in 500 cc. of distilled water, 500 cc. of 5 per cent ammonium hydroxide is slowly added with vigorous stirring. The precipitate is washed by decantation until free of ammonia The suspension is diluted to 1 liter

*Procedure.*—To determine the minimum amount of adsorben trequired the following standard technique is employed 1 cc of the sus-

* Much of the material on adsorbents is the result of studies made by Dr Mario Stefanini and Miss Clara Hussey

pension of adsorbent is transferred to a test tube and centrifuged for 5 minutes to packed the agent. The water is poured off, the tube is drained and dried with a filter paper One cc. of oxalated plasma is added to the pack adsorbent and the two mixed by stirring with a small glass rod for 3 minutes and then allowed to stand 2 more minutes The adsorbent is removed by centrifugation at 2500 r p m for 5 minutes. The prothrombin time of the adsorbed plasma is determined by the one-stage method (Test 8). If no clot forms in 5 minutes, complete adsorption is assumed. By preparing progressive dilutions of the standard stock solution, one can determine the minimum quantity of the suspension that will bring about complete adsorption of prothrombin. In table 20 the adsorbent capacity for

TABLE 20.—THE MINIMUM CONCENTRATION OF ADSORBENT REQUIRED FOR COMPLETE REMOVAL OF PROTHROMBIN FROM OXALATED PLASMA

| *Adsorbent* | *Human Plasma* | *Rabbit Plasma* |
|---|---|---|
| $Ca_3(PO_4)_2$ | 0 005 M * | 0 012 M |
| $BaSO_4$ | 0 150 M. | 0 200 M |
| $Al(OH)_3$ | 0 060 M | 0 120 M |
| $Mg(OH)_2$ | 0 600 M. | 0 900 M |

* The amount of adsorbent in 1 cc of suspension necessary to remove prothrombin completely from 1 cc of oxalated plasma

prothrombin of four common agents when applied to human and rabbit plasma is recorded

*Application and Interpretation* – The adsorbents for prothrombin have been valuable in isolating this compound from plasma By means of tricalcium phosphate, prothrombin is completely removed from oxalated or native plasma, and is quantitatively recovered by elution with sodium citrate This observation has been utilized for developing a new method for the quantitative determination of prothrombin (Test 9) and also for the isolation of prothrombin in concentrated form to treat hypoprothrombinemia Barium sulfate behaves similarly to tricalcium phosphate, but is less efficient Aluminum hydroxide and magnesium hydroxide, on the contrary, are unsatisfactory for isolating prothrombin since sodium citrate does not quantitatively elute the agent from these adsorbents

None of the adsorbents is specific for prothrombin, but tricalcium phosphate can be considered as approaching that property The concentration required to deprothrombinize plasma is so small that only a trace of labile factor and less than 3 per cent of the fibrinogen are removed Barium sulfate likewise removes very little labile factor, but it does decrease the fibrinogen of the treated plasma about 10 per cent Aluminum hydroxide and magnesium hydroxide in the con-

centrations necessary to adsorb prothrombin completely, remove large amounts of fibrinogen (30 to 70 per cent) and also markedly diminish the content of labile factor. Deprothrombinized plasmas, *i.e*, plasmas treated with an adsorbent for prothrombin, are useful for studying coagulation reactions, but their limitations should be recognized. A plasma obtained after treatment with an adsorbent should not be considered identical in behavior to a solution of fibrinogen. It still retains wholly or in part all the recognized clotting factors except prothrombin and prothrombinogen

## Test 20

### THE ASSAY OF VITAMIN K

*Principle.**—Vitamin K is essential for the synthesis of prothrombin. It is logical therefore that it should be assayed by determining either the minimal daily amount required to maintain a normal prothrombin level of the blood, or the quantity which will elevate the prothrombin of an animal severely depleted of vitamin K to normal in a fixed period of time

The protective type of bioassay is carried out on newly hatched chicks kept on a vitamin K-free diet The material to be tested is fed at different levels and the prothrombin time performed at the end of a fixed period of time. For the curative type of assay, the chick likewise is very satisfactory as the test animal and the material may either be fed or injected In the writer's laboratory, a new assay procedure employing the dog as the test animal has been developed A cholecystnephrostomy is performed With the complete drainage of the bile into the pelvis of the kidney, the absorption of vitamin K from the intestine becomes very defective, and as the vitamin K stores of the animal become depleted a hypoprothrombinemia develops The response of the prothrombin concentration in such an animal to injected vitamin K is utilized as the basis of assay, and natural vitamin $K_1$ is used as the standard of reference

*Equipment and Reagents*

Electrically heated brooder with wire mesh floor (Purchased at Sears-Roebuck and Co)

Syringes—1 cc silicone-coated

Needles No 27

Test tubes 8 × 75 mm silicone-coated

Vitamin K-free ration

2000 gm vitamin-free casein

* On the principle as outlined, the writer devised a method of assay in 1936 [Quick, A J The coagulation defect in sweet clover disease and in the hemorrhagic chick disease of dietary origin A consideration of the source of prothrombin, Am J Physiol, *118*, 260, 1937]

Later Almquist and Klose [Almquist, H J, and Klose A A Determination of the anti-hæmorrhagic vitamin, Biochem J *33*, 1055 1939] adopted the prothrombin time in their assay procedure and this became the basis of the method given in Methods of Analysis, Assoc Off Agr Chem, 6 ed, Washington, D C, 1945 This method is relatively crude and lacks the high degree of sensitivity that the procedures here described have

1200 gm. brewer's yeast
6700 gm. ground rice
100 gm salt mixture.

Immediately before feeding, 1 cc. of cod liver oil (U S.P.) is thoroughly mixed with 100 gm. of the ration. The casein and brewer's yeast were obtained from Nutritional Biochemical Corp, Cleveland, Ohio The salt mixture consists of:

| | |
|---|---|
| 48 gm. calcium carbonate | 1.4 gm potassium chloride |
| 48 gm. sodium chloride | 1.0 gm. manganous sulfate |
| 1 4 gm ferrous sulfate | 0 2 gm copper sulfate |

Thromboplastin (dehydrated chicken brain): The material is prepared exactly as rabbit brain thromboplastin (Test 7). To prepare the reagent, 0 15 gm. are mixed with 5 cc. saline solution and incubated at 50° C for 20 minutes

Stock solution of natural vitamin $K_1$ 200 mg. of vitamin $K_1$ (Merck) is dissolved in 100 cc. of 95 per cent alcohol. This solution must be protected from light. Generally 1 cc. of the stock solution is diluted to 100 cc with saline (1 cc. = 20 gammas). The diluted solution must be well shaken since the oily vitamin separates out as a milky suspension

Stock solution of 2-methyl-1,4-naphthoquinone (menadione)· 200 mg of the crystalline compound is dissolved in 100 cc. of alcohol. The solution is protected from light One cc of the stock solution is diluted to 100 cc with saline (1 cc = 20 gammas). The dilute solutions of vitamin $K_1$ and menadione are prepared fresh just before they are administered

*Procedure* —The chick protective assay method [1] Newly hatched chicks are placed in an electrically heated brooder with a wire mesh floor Drinking water containing 0 1 per cent benzoic acid to inhibit growth of bacteria is supplied The vitamin K-free ration is fed *ad libitum*. For the protective assay method, the material to be tested is dissolved or suspended in water or dilute alcohol By means of a serological pipette 0 05 to 0 3 cc is given orally Care must be taken to insert the pipette deep into the throat The chick is not allowed to eat or drink for 20 to 30 minutes after the material is given The substance to be tested is given once daily For each dose 3 to 5 chicks are used, and the doses are so graduated that the minimal effective amount can be determined

At the end of 10 or 11 days the prothrombin time of each chick is determined For this a silicone-coated 1 cc syringe and a No 27 needle are used Blood is drawn from the wing vein From 0 3 to 0 5 cc. of blood is collected and mixed with 0 1 M sodium oxalate in the proportion of 9 to 1 It is best to use small silicone-coated test tubes

(8 × 75 mm) kept in an ice bath. Enough plasma is obtained for 2 or more determinations of prothrombin. The procedure for the prothrombin time is exactly as outlined in Test 8 except that active dehydrated chicken brain is used as the thromboplastin reagent.

As reference standards, menadione and vitamin $K_1$ (Merck) are employed The results recorded in table 21 serve as a guide

TABLE 21 —COMPARATIVE POTENCY OF 2-METHYL-1,4 NAPHTHOQUINONE AND VITAMIN $K_1$ WHEN TESTED ON THE CHICK

| | *Amount given daily* | *Chick 1* | *1* | *2* | *3* | *5* | *Average* | *Prothrombin concentration* |
|---|---|---|---|---|---|---|---|---|
| | | *Prothrombin time on 11th day* | | | | | | |
| | *gamma* | *sec* | *sec* | *sec* | *sec* | *sec* | *sec* | |
| 2 Methyl-1,4-naphthoquinone | 0 | 59 | 100 | 54 | 55 | 63 | 66 | 4 |
| | 0.5 | 18 | 16 | 14 | 27 | 25 | 20 | 18 |
| | 1.0 | 14 | 14 | 18 | 14 | 15 | 15 | 30 |
| | 1.5 | 13 | 11 | 12½ | 12 | 13½ | 12 | 60 |
| | 2.0 | 11 | 11 | 11 | 11 | 11 | 11 | 100 |
| Vitamin $K_1$ | 0 | 33 | 52 | 54 | 47 | 55 | 48 | 5 |
| | 1.25 | 13 | 16 | 18 | 13½ | 14 | 15 | 30 |
| | 2.5 | 12 | 13 | 11 | 13½ | 11½ | 12 | 60 |
| | 3.75 | 11 | 11 | 11 | 11 | 11½ | 11 | 100 |
| | 5.0 | 11½ | 11 | 11½ | 10½ | 11 | 11 | 100 |

The chick curative assay method [1] The procedure is the same as that of the protective method except that all of the chicks are kept on the vitamin K-free diet At the end of 10 to 11 days, at which time the prothrombin is reduced to 5 per cent or less, the assay is carried out. If the material is dissolved in water or alcohol, it is appropriately diluted, and 0.2 cc is injected intravenously With a little skill, blood can be withdrawn for the prothrombin determination, and the material injected with the same venipuncture Exactly 4 hours later, a sample of blood is taken and analyzed for prothrombin The object is to determine the smallest amount which will reduce the prothrombin time to 11 seconds, which is the normal or 100 per cent level of the chicks It has been found that 4 to 5 gammas of 2-methyl-1,4-naphthoquinone (menadione) will restore the prothrombin level of a severely vitamin K-depleted chick in 4 hours Therefore the amount of any unknown material which reduces the prothrombin time to normal in 4 hours contains the equivalent of 4 to 5 gammas of menadione The assay can also be made by administering the material orally, in which case the prothrombin is determined in 24 instead of 4 hours

The dog curative assay method A cholecystnephrostomy is performed according to the method of Kapsinow, Engle and Harvey,[2]

1200 gm. brewer's yeast.
6700 gm. ground rice.
100 gm. salt mixture

Immediately before feeding, 1 cc. of cod liver oil (U.S.P.) is thoroughly mixed with 100 gm of the ration The casein and brewer's yeast were obtained from Nutritional Biochemical Corp., Cleveland, Ohio The salt mixture consists of.

| | |
|---|---|
| 48 gm calcium carbonate | 1 4 gm. potassium chloride |
| 48 gm sodium chloride | 1 0 gm. manganous sulfate |
| 1.4 gm. ferrous sulfate | 0.2 gm. copper sulfate. |

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Stock solution of 2-methyl-1,4-naphthoquinone (menadione). 200 mg of the crystalline compound is dissolved in 100 cc of alcohol The solution is protected from light One cc of the stock solution is diluted to 100 cc with saline (1 cc = 20 gammas). The dilute solutions of vitamin $K_1$ and menadione are prepared fresh just before they are administered

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At the end of 10 or 11 days the prothrombin time of each chick is determined For this a silicone-coated 1 cc syringe and a No 27 needle are used. Blood is drawn from the wing vein From 0 3 to 0 5 cc. of blood is collected and mixed with 0 1 M sodium oxalate in the proportion of 9 to 1 It is best to use small silicone-coated test tubes

normal by a minimal dose, the decrease in prothrombin is slow and gradual

The available experimental findings are in favor of the assumption that vitamin K becomes a part of the enzyme which synthesizes prothrombin. One can postulate that it functions as the prosthetic group which can be expressed by the equation

$$Æ + K_1 \rightleftharpoons ÆK_1 \qquad \begin{array}{l} Æ = \text{apoenzyme} \\ ÆK_1 = \text{prothrombin forming enzyme} \end{array}$$

The concentration of apoenzyme is not affected by lack of vitamin $K_1$ for on supplying the vitamin, the synthesis of prothrombin is resumed so promptly that the normal level is restored in 4 hours Significantly, 4 hours is the time required both by the dog and the chick, and even if the amount of vitamin $K_1$ above the minimum effective dose is given it does not shorten the time

Prothrombin is utilized or destroyed at such a rate that approximately 80 per cent of the amount circulating in the blood disappears in 24 hours The prothrombin level of the blood therefore is a resultant of the rate of synthesis and the speed of destruction The amount of vitamin K required for the total enzymatic system is very small As already pointed out, 100 gammas suffice to restore completely the prothrombin of a 14 kg dog On that basis, an adult human requires about 0 5 mg of vitamin $K_1$ and for maintenance the daily need is probably less than one-fifth of that amount

In view of the extremely small daily requirement of vitamin K, the concentration of this compound in foods is clinically of little importance Except in the newborn, a vitamin K deficiency in the human purely on a dietary basis is extremely unlikely, since the intestinal bacteria supply some of the vitamin, and furthermore, there is a considerable storage of the fat soluble vitamins including K

A comparison of the potency of synthetic compounds having vitamin K activity with the natural product can be done most reliably using a dog that is markedly deficient in vitamin K A study comparing 2-methyl-1,4-naphthoquinone with natural vitamin $K_1$ is presented in figure 15 While it is generally accepted that 2-methyl-1,4-naphthoquinone is as effective as vitamin $K_1$, the results of this study show that the compound, to the contrary, is not nearly as potent Interestingly, the prothrombin level in the absence of natural vitamin $K_1$ can only be brought to 40 per cent with 2-methyl-1,4-naphthoquinone even when excessive amounts are administered Apparently there is formed the enzyme ÆM (M = menadione) which is far less efficient than the truly physiological one, which is $ÆK_1$ The result is that the rate of synthesis is such that only a level of

After a period of time which may vary from several weeks to four months or more, the prothrombin of the blood decreases. In some dogs, the prothrombin drops to a very low level and this is accompanied by a pronounced bleeding tendency. In the majority of dogs, however, the prothrombin concentration drops but reaches a fixed level. These dogs apparently retain the capacity to absorb a small but constant amount of vitamin K from the intestines The dogs which develop a severe hypoprothrombinemia are exceedingly sensitive to minute amouts of natural vitamin $K_1$ It can be seen from table 7, that 125 gammas of vitamin $K_1$ which is 9 gammas per kg. of body weight brought the prothrombin from a level of less than 0.5 per cent to normal in 4 hours, whereas a slightly smaller dose (7 gammas per kg of body weight) only brought it to 50 per cent in 4 hours Since these animals do not absorb vitamin K from the intestines, any material to be assayed must be given intravenously.

*Application and Interpretation* —The assay for vitamin K activity has two general uses The first is the analysis of various food stuffs for their vitamin K content, the second is to determine the comparative potency of synthetic compounds such as 2-methyl-1,4-naphthoquinone with natural vitamin $K_1$ For the first purpose, the chick methods are the most suitable, whereas the more reliable procedure to determine relative potency of compounds having vitamin K activity is the dog assay method

Before these applications can be adequately discussed, it is necessary to consider the function of vitamin K in the synthesis of prothrombin The two most likely ways that vitamin K may function are it becomes a part of the prothrombin molecule, or it enters into the enzymatic structure that produces prothrombin The evidence clearly indicates that the second postulate is the more likely As already pointed out, 100 gammas of vitamin $K_1$ is enough to restore completely the prothrombin of a dog weighing 14 kg The volume of blood can be estimated to be over 1000 cc If vitamin $K_1$ becomes a part of the prothrombin molecule, 100 gammas with its relatively high molecular weight (450) would have to be contained in the prothrombin of 1000 cc. of blood Since the molecular weight of prothrombin is 140,000 according to Seegers and Ware,[3] the total prothrombin would be $140{,}000 \div 450 \times 100$ gammas or approximately 30 mg Isolation studies have shown that the amount of prothrombin is several times larger

As further evidence that vitamin $K_1$ does not become a part of the prothrombin molecule, is the observation that when the synthesis of prothrombin is stopped, the level drops rapidly, whereas if vitamin K is withheld after the level of prothrombin has been brought to

normal by a minimal dose, the decrease in prothrombin is slow and gradual

The available experimental findings are in favor of the assumption that vitamin K becomes a part of the enzyme which synthesizes prothrombin. One can postulate that it functions as the prosthetic group which can be expressed by the equation:

$$Æ + K_1 \rightleftharpoons ÆK_1 \qquad \begin{array}{l} Æ = \text{apoenzyme} \\ ÆK_1 = \text{prothrombin forming enzyme} \end{array}$$

The concentration of apoenzmye is not affected by lack of vitamin $K_1$ for on supplying the vitamin, the synthesis of prothrombin is resumed so promptly that the normal level is restored in 4 hours. Significantly, 4 hours is the time required both by the dog and the chick, and even if the amount of vitamin $K_1$ above the minimum effective dose is given it does not shorten the time

Prothrombin is utilized or destroyed at such a rate that approximately 80 per cent of the amount circulating in the blood disappears in 24 hours. The prothrombin level of the blood therefore is a resultant of the rate of synthesis and the speed of destruction The amount of vitamin K required for the total enzymatic system is very small As already pointed out, 100 gammas suffice to restore completely the prothrombin of a 14 kg. dog On that basis, an adult human requires about 0 5 mg. of vitamin $K_1$ and for maintenance the daily need is probably less than one-fifth of that amount

In view of the extremely small daily requirement of vitamin K, the concentration of this compound in foods is clinically of little importance Except in the newborn, a vitamin K deficiency in the human purely on a dietary basis is extremely unlikely, since the intestinal bacteria supply some of the vitamin, and furthermore, there is a considerable storage of the fat soluble vitamins including K

A comparison of the potency of synthetic compounds having vitamin K activity with the natural product can be done most reliably using a dog that is markedly deficient in vitamin K A study comparing 2-methyl-1,4-naphthoquinone with natural vitamin $K_1$ is presented in figure 15. While it is generally accepted that 2-methyl-1,4-naphthoquinone is as effective as vitamin $K_1$, the results of this study show that the compound, to the contrary, is not nearly as potent. Interestingly, the prothrombin level in the absence of natural vitamin $K_1$ can only be brought to 40 per cent with 2-methyl-1,4-naphthoquinone even when excessive amounts are administered Apparently there is formed the enzyme ÆM (M = menadione) which is far less efficient than the truly physiological one, which is $ÆK_1$ The result is that the rate of synthesis is such that only a level of

40 per cent can be maintained. It is also apparent that 100 gammas of the synthetic compound is far less effective than the same amount of the natural vitamin $K_1$. Although the assay method using chicks indicates that on a molecular basis menadione is less potent than vitamin $K_1$, the striking difference is only brought out with dogs having a cholecystnephrostomy.

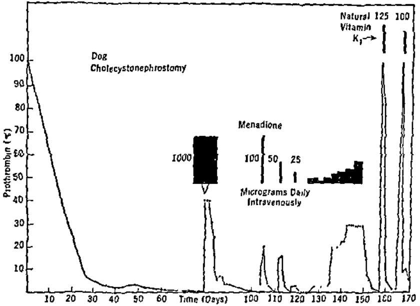



FIG. 15 – The comparative potency of 2-methyl-1,4-naphthoquinone (menadione) and natural vitamin $K_1$ for correcting the hypoprothrombinemia caused by vitamin K deficiency.

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* To Dr. George Collentine belongs the credit of preparing the cholecystnephrostomy dogs as well as doing much of the laboratory work with these dogs both on vitamin K and Dicumarol.

## Test 21

# DETERMINATION OF THE POTENCY OF PROTHROMBINOPENIA-INDUCING AGENTS

*Principle* —Certain compounds of which 3,3'-methylene-bis-(4-hydroxycoumarin) commonly known as Dicumarol, is the most important, have the power to decrease the prothrombin of the blood To test the potency of these drugs, it is essential to have a standard test animal, a controlled diet, and a reliable method for determining prothrombin Resistance to the drug may be inherent in the animal itself, or be due to stored vitamin K Chickens, for instance, are strikingly resistant to the action of Dicumarol even when deficient in vitamin K. Dogs on the other hand are less susceptible to Dicumarol than are rabbits, but when their store of vitamin K becomes depleted, they are very markedly affected by this drug Thus, a dog that has had a cholecystnephrostomy becomes a suitable test animal as soon as a slight drop in prothrombin becomes manifest Rabbits and rats readily become deficient in vitamin K and therefore under those conditions show a relatively high sensitivity to Dicumarol The writer[1] as early as 1936 found that toxic sweet clover hay, which reduced the prothrombin of a rabbit to 20 per cent of normal in 48 hours, became ineffective when mixed with 5 per cent alfalfa meal This finding was the first to illustrate the antagonistic action of vitamin K to the toxic principle in spoiled sweet clover hay Campbell *et al*,[2] wisely fasted rabbits for 24 to 48 hours before testing their spoiled sweet clover hay material

*Apparatus and Reagents* —The same as for Test 8

*Procedure* —The most suitable test animals for assaying prothrombinopenic-producing drugs are rabbits and dogs since in these animals blood can readily be obtained by venipuncture It is very important that the animals be kept on a standard diet Rabbits fasted 48 hours are not only more sensitive to Dicumarol, but their response is more uniform Campbell *et al*,[2] emphasized that certain rabbits are very resistant to Dicumarol and that this is inherited as a Mendelian characteristic While there may occasionally be found a rabbit fairly resistant to Dicumarol, most rabbits when kept on a standardized diet respond with remarkable uniformity to the drug On picking 5 rabbits at random, one can obtain results as follows

| Rabbit | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Prothrombin time before given Dicumarol* | 9 | 8 | 8 | 8 | 8 |
| Prothrombin time 24 hours after given Dicumarol | 18 | 18 | 17 | 17 | 17 |

* 5 mg per kilo of body weight

It should be noted that these rabbits did not have a prothrombin time of 6 seconds which is the normal when they receive an adequate amount of vitamin K.

In normal dogs the response to Dicumarol is fairly uniform as shown in figure 16 It should be noted that the size of the dose does not

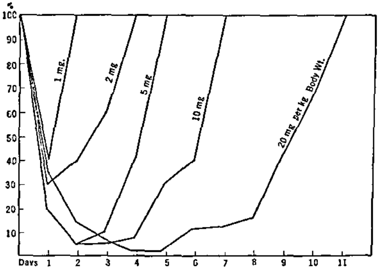



FIG 16 —The effect of the size of a single dose of Dicumarol given intravenously on the prothrombin level of a dog (By permission of the Journal of Biological Chemistry)

hasten the drop in prothrombin, but prolongs the period of recovery

Prothrombin concentration is determined exactly as outlined in Test 8 Dehydrated rabbit brain thromboplastin is entirely satisfactory for human, rabbit and dog blood, but is unsuited for rats or guinea pig blood

*Application and Interpretation* —There are few drugs which are administered with such recklessness and abandonment as is Dicumarol It is most fortunate that man is not as sensitive to this drug as is the cow That there have been deaths from hemorrhage caused by Dicumarol is no surprise, the wonder is that there have been so few It is impossible to estimate how many patients in the country are receiving the drug without any laboratory control One can state, however, with authority that in a far greater number of patients who are given the drug, therapy is controlled by prothrombin time

determinations which are so crude and unreliable that one can rightly say the patient is merely receiving token protection Perhaps an equal number of patients are receiving the drug in such homeopathic doses that any beneficial effect belongs in the realm of psychosomatic medicine. Many conscientious clinicians bemoan the fact that the *whole* problem is in such a state of confusion that the results of one laboratory or clinic cannot be compared with those of another

The writer feels that the beginning of the trouble can be traced to Dr. Link and his students. After their brilliant work in isolating and making Dicumarol available, they befuddled, according to the writer's opinion, the issue of the determination of prothrombin in controlling Dicumarol therapy The crux of the matter can be found in their statement· "The assay has been put on a *strictly differential basis*, which eliminates the necessity of obtaining absolute values, since the pathic plasma is compared directly with plasma from the same rabbit in the normal state."[2] The writer had already shown that the determination of prothrombin could be made in absolute values, and neither time nor progress has thus far made it necessary for him to alter this statement But once the strict insistence on absolute values was relaxed, a flood of modifications of the one-stage method appeared in the literature A critical discussion of these modifications is presented under Test 8 There seems to be no prospect of ending the present confusion except to insist that the determination of prothrombin be made *in absolute values* and not on a differential basis

Recent work in the writer's laboratory, especially by Dr Collentine on dogs with a cholecystnephrostomy, has yielded important data having a bearing on the nature of the action of Dicumarol and its relation to vitamin K Dicumarol has no direct action on prothrombin The drug stops the synthesis of prothrombin, but the actual decrease of the latter results from the body's normal consumption of this agent The Dicumarol merely prevents its replacement or replenishment Dicumarol can be looked upon as a true antivitamin K, i e, it can combine with the apoenzyme Æ, and thus form an inactive enzyme When Dicumarol is given to an animal with a good store of vitamin K one can postulate the reaction given in figure 17

The greater the amount of Dicumarol, the more ÆD is formed, and the less will be the amount of functioning enzyme ÆK If Dicumarol is given to an animal deficient in vitamin K, the effect of even a small dose is pronounced as shown by the results presented in figure 18

In figure 17 the effect of salicylates is illustrated The salicylates have an extremely weak affinity for the apoenzyme, therefore a hypoprothrombinemia as the result of salicylate therapy is infrequent

| Rabbit | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Prothrombin time before given Dicumarol* | 9 | 8 | 8 | 8 | 8 |
| Prothrombin time 24 hours after given Dicumarol | 18 | 18 | 17 | 17 | 17 |

* *5 mg per kilo of body weight.*

It should be noted that these rabbits did not have a prothrombin time of 6 seconds which is the normal when they receive an adequate amount of vitamin K

In normal dogs the response to Dicumarol is fairly uniform as shown in figure 16. It should be noted that the size of the dose does not

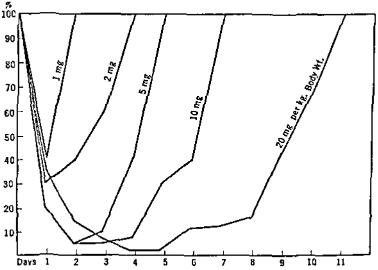



FIG. 16 —The effect of the size of a single dose of Dicumarol given intravenously on the prothrombin level of a dog (By permission of the Journal of Biological Chemistry)

hasten the drop in prothrombin, but prolongs the period of recovery

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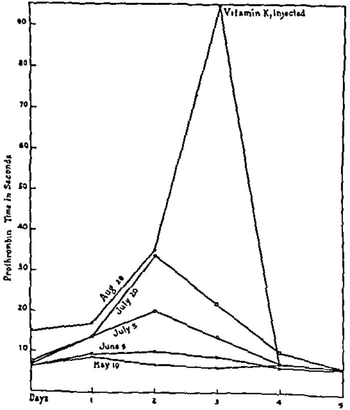



FIG 18—The effect of stored vitamin K on the sensitivity of the animal to Dicumarol The dog was well-nourished at the time of the operation (May 12) Following cholecystnephrostomy, the dog was no longer able to absorb fat-soluble vitamins from the intestines, therefore the store of vitamin K was gradually diminished The test dose of Dicumarol was 1 mg per kilo of body weight given intravenously in each instance A small amount of vitamin $K_1$ (10 gammas per kilo of body weight) restored the prothrombin level to normal in 24 hours

ency, in ulcerative lesions, and after certain operations such as on the brain or spinal cord. In addition to these, pregnancy is also an important contraindication since the fetus is more susceptible than the mother and may suffer hemorrhage even though the maternal prothrombin level is in the therapeutic range While the initial dose is generally 200 to 300 mg, it should be remembered that some individuals will show an abnormally great response while others will show little hypoprothrombinemic effect. It is therefore necessary to determine the prothrombin time after 24 hours in order to know

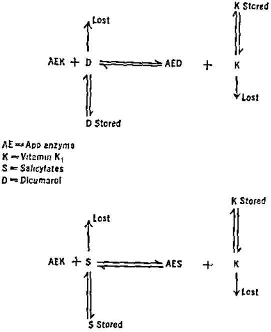



FIG 17 —The probable action of Dicumarol and of salicylates on the prothrombin-producing mechanism

By depleting the store of vitamin K in the dog by cholecystnephrostomy, the effect of salicylates can readily be illustrated

| Days | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Prothrombin time | 12 | 12 | 18 | 15 | 15 |

A daily dose of 0 05 gm per kg was given daily intravenously

It may well be, as Dr Link suggests[3] that among the poor and malnourished, the intake of vitamins K and C may be inadequate, and it can be concluded that this may predispose such individual to a greater sensitivity to salicylates It seems good medical practice and sound economy to see that when such patients require salicylates that their nutrition be improved rather than to depend on commercial preparations of salicylates fortified with a synthetic compound having vitamin K activity Mention should also be made that salicylates can cause bleeding by producing thrombocytopenia which is totally unrelated to hypoprothrombinemia

In the control of Dicumarol therapy, a few simple rules must be followed As Allen and his associates[4] have pointed out, the use of the drug is contraindicated in renal insufficiency, in hepatic disease, in nutritional deficiencies, in blood dyscrasias with a bleeding tend-

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what the size of the second dose should be. The aim is to reduce the prothrombin level to about 20 to 25 per cent of normal. The maintenance dose may vary from 25 to 100 mg. or even more daily. In some patients the maintenance dose is very constant and they may require only one determination every 7 or 10 days after they are standardized But before a patient is standardized daily laboratory studies are necessary. Every patient receiving Dicumarol should be instructed to observe his urine, since hematuria for some curious reason is one of the earliest and most common signs of bleeding induced by Dicumarol

While it must be granted that some modifications of the one-stage prothrombin procedure are used successfully in the clinical control of Dicumarol therapy, it should be pointed out that with little extra effort and no additional expense the method as outlined (Test 8) can be carried out. Not only will the laboratory gain by getting data that are accurate, but its results can be compared with various laboratories that are now using the unmodified one-stage procedure. An additional advantage is that any laboratory that uses the original procedure will have no difficulty doing the prothrombin consumption test, which will probably become a routine laboratory test.

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